12-2013

# The Development of an In Vivo Microdialysis Collection Method of Cytokines from Brain Tissue

Anthony W. Herbaugh
*University of Arkansas, Fayetteville*

# The Development of an In Vivo Microdialysis Collection Method of Cytokines from Brain Tissue

# The Development of an In Vivo Microdialysis Collection Method of Cytokines from Brain Tissue

A thesis submitted in partial fulfillment
of the requirements for the degree of
Master of Science in Cell and Molecular Biology

by

Anthony W. Herbaugh
Hendrix College
Bachelor of Arts in Biology, 2005

December 2013
University of Arkansas

This thesis is approved for recommendation to the Graduate Council.

Dr. Julie Stenken

Thesis Director

Dr. Ingrid Fritsch

Committee Member

Dr. Wayne Kuenzel

Committee Member

**Abstract**

In this thesis, different methods to improve the microdialysis collection procedure for cytokines from brain tissue are presented. The first method was based on stopped flow and results indicating that no significant difference in relative recovery between stopped flow and continuous flow are shown. The second method is an antibody bead-based enhancement method. With the antibody bead-based method, a 3.5 fold increase in the collected concentrations of Chemokine (C-C motif) Ligand 2 (CCL2) were observed. However, there was no significant increase in the in vivo collection efficiency of Interleukin-6 (IL-6) using the antibody enhancement. Finally the development of an in-house created bead assay is discussed for the enhanced collection and quantification for molecules that do not have a commercially available bead-based immunoassay kit. The in-house assay when compared to a commercially available kit for CCL2 showed that a successful assay can be made using the technique employed. Using this method a bead based assay could be made for molecules that do not have these commercially available kits.

**Acknowledgements**

I would like to thank Dr. Julie Stenken for all of her support, understanding, and guidance though all of these years.

I would like to thank Dr. Jia Duo for his instruction and teaching of methods and instrumentation.

I would like to thank the rest of the members of Dr. Stenken's lab for all of their support and collaboration through the years.

Finally I would like to thank the NIH for their funding and support of this research.
NIH EB-001440 and NIH NS-075874

**Dedication**

I would like to dedicate this thesis to my wife Nancy. Without her by my side to help me through the darkest parts none of this would be possible. I would also like to thank my parents for everything they have done for me through my whole life, my father for teaching me the joys of science and to my mother for always caring and supporting me even when I did not believe in myself.

**Table of Contents**

**List of Figures and Graphs**

**List of Abbreviations and Symbols**

aECF – Artificial Extracellular Fluid

CCL2 - Chemokine(C-C motif) Ligand 2

CNS - Central Nervous System

ECF - Extracellular Fluid

EDC - 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride

EE – Extraction Efficiency

ELISA - Enzyme-linked Immunosorbent Assay

FBS - Fetal bovine serum

IFN-γ - Interferon-gamma

IL-6 - Interleukin-6

MES - 2[N-morpholino]ethansulfonic acid

MCP-1 - Monocyte Chemoattractant Protein-1, See also CCL2

MWCO - Molecular Weight Cut Off

NHS - N-hydroxysuccinimide

PBS - Phosphate buffered saline

PES - polyethersulfone membrane

RIA - Radioisotope Immunoassay

RT-PCR - Real Time Polymerase Chain Reaction

SAPE - Streptavidin, R-phycoerythrin conjugate

*Symbols:*

$C_{inlet}$ – Concentration going into Inlet

$C_{outlet}$ – Concentration coming out of the Outlet

$C_{sample\infty}$ - Concentration of the sample at the farthest point from the probe

$D_d$ – Diffusion Coefficient of Solute in Dialysate

$D_m$ – Diffusion Coefficient of Solute in Membrane

$D_q$ – Diffusion Coefficient of Solute in Tissue(or Sample Media)

$L$ – Length of Probe

$Q_d$- Flow Rate

$r_\alpha$ – Radius of Cannula

$r_\mathrm{i}$ – Radius of Inner Canula

RR – Relative Recovery

$R_d$ – Resistance of Dialysate

$R_m$ – Resistance of Membrane

$R_{sm}$ – Resistance of Sample Media

$R_{ts}$ – Resistance of Tissue

$\Phi_q$ – Water Fraction of Tissue

$\Phi_m$ – Water Fraction of Membra

## 1.0 Introduction

Within the brain, communication between cells occurs via the release of different chemicals from presynaptic neurons through the synapse to receptors on the post-synaptic neuron. These chemicals are classified in different ways and include low molecular weight compounds such as dopamine and serotonin to large molecular weight proteins. Dopamine and serotonin represent a class of chemicals referred to as the classical neurotransmitters. Neuropeptides such as leucine-enkephalin and neuropeptide Y are considered by some to be neurotransmitters and by others to be neuromodulators. A third class of neuromodulators is the cytokine proteins.

Cytokines are a large class of proteins that are secreted not only by different cells in the immune system, but also from different cells within the central nervous system (CNS). Cytokines are emitted in response to immunological threat or injury and play an important role in development and homeostasis. Additionally, cytokines are known to work within a network of different proteins affecting different functions commonly referred to as, the "cytokine network."

### 1.1 History of Neurochemical Collection or Detection in Brain.

The existence of classical neurotransmitters and some neuropeptides was known and verified long before there were methods to detect the presence of these chemicals directly within the brain extracellular space (ECF) in living beings. By the late 1960s and early 1970s there was a tremendous interest in creating chemical analysis tools that would allow for the collection or detection of neurotransmitters.

### 1.1.1 Whole Brain

A common method of obtaining proteins from brain tissue is the use of a biopsy, where a small section of tissue is taken out and analyzed. In addition to small sections whole brain biopsy is also used where the whole brain is taken out to analyze. This biopsy method is most limited in that it only gives a single measurement at the time the sample was taken; changes in protein levels that may occur throughout the duration of an injury or disease cannot be analyzed. Not being able to analyze protein changes in a temporal manner requires many additional animals.

### 1.1.2 *In Vivo* Electrochemistry

By the mid-1970s, Ralph Adams and colleagues developed the technique of *in vivo* voltammetry to measure the electrochemically active catecholamines.[1] Around the same time, Ungerstedt began to use dialysis tubes with attached inlet and outlet tubing as an "artificial capillary" to allow for collection of classical neurotransmitters.[2]

Since electrochemical methods are only able to detect chemicals that are redox active, this analysis tool is limited in the sampling of neuromodulators since there are many more neuromodulators than the biogenic amines that are not redox active including amino acid neurotransmitters such as glutamate and GABA. Cytokines are included in the group that is not redox active. For this reason, with respect to *in vivo* cytokine detection, electrochemistry is not an appropriate choice for measurement.

### 1.1.3 Push Pull

At the same time that electrochemical methods were being developed for catecholamine detection, late 1960s, the use of a technique called push-pull perfusion was being developed and described for collecting samples from the ECF in awake animals.[3] In this technique a hollow tube with an open end is inserted into the tissue. A perfusion fluid is then pumped into the tube and out into the tissue. After a short time, this fluid is then pulled back through the tube using a vacuum allowing sample collection. Push-pull has been used to collect peptides within the brain.[4, 5] Push-pull however has limitations as well. There are concerns within the research community regarding how much damage is done to the tissue by pushing the fluid into it. Kottegoda et al.[6] show that the use of low flow rates limits the amount of damage done by push-pull perfusion. However Myers et al.[7] have compared push-pull to microdialysis and found that push-pull caused more tissue damage. Push-pull is also not an analytically clean method of collection as microdialysis is thus further sample preparation and separation is often needed before detection leading to sample loss and degradation. Only recently has this problem been overcome by direct coupling of the separation and detection in-line with the push-pull.[8]

### 1.1.4 Microdialysis

Microdialysis is a sampling technique that allows the collection of molecules from the tissue of interest. Microdialysis sampling uses an outer semi-permeable membrane that is size selective with a defined molecular weight cutoff (MWCO). Solutes that are smaller than the membrane MWCO will diffuse through the membrane. Solutes that diffuse across the membrane into a perfusion fluid that carries the molecule out of the probe allowing for sample collection and analysis. Once collected solutes can then be further analyzed without additional sample preparation steps. An in-depth discussion of microdialysis sampling is provided in Section 1.3.

## 1.2 Cytokine Signaling within the Brain

Cytokines are signaling proteins released by cells of the immune system - macrophages, neutrophils, basophils, eosinophils, and lymphocytes.[9] Cytokines are used as signaling proteins to regulate and control the immune response and inflammation.[10] Cytokines can vary in size and structure from 5 to 80 kDa.[11] Cytokines can only act on those cells that express receptors for cytokines. Cytokines have been shown to have autocrine, paracrine and endocrine function between immune cells.[12]

The binding of the cytokine to its receptor will trigger a secondary messenger cascade that will regulate transcription factor and vesicle releasing factors. This way cytokines regulate the production of further proteins in the immune cells and the release of further cytokines.

Because the cytokine system is so extensive this section will only go into those specific cytokines researched with in this thesis and only their function within the brain. Chemokine(C-C motif) Ligand 2(CCL2 or MCP-1) has been shown to alter the permeability of the blood brain barrier and regulate vascular control.[13, 14]

### 1.2.1 Cellular Origins

Within the brain, cytokines have been found within nearly every type of cell; glial, neuron, and astrocytes. Cytokine receptors have also been found in almost every region of the brain.[15,16] The exact reason for the presence of these receptors on neurons is unknown, but it has been proposed that cytokines and chemokines act as a third type of neuronal chemical communication group.[17]

Initial work into identifying cytokines and their receptors within brain tissue has been performed mainly with two techniques. Real time polymerase chain reaction (RT-PCR)[18] has been used to identify cell types within different brain regions that are involved in the cytokine network. PCR it is important to point out does not actually detect the protein but the expression of the protein, to actually detect cytokines or their receptor proteins requires immunohistochemistry approaches.[19] These techniques identify which cells have cytokines or their receptors. However since cytokine function is concentration dependent in many of its roles it is important to get a measurement of the actual protein to determine effects on the system.[20, 21] Limited time lapse studies done on homogenized tissue has shown that cytokine concentration is dynamic in vivo.[9, 11] These techniques can only offer a single time point examination of the concentration. The same problem occurs with a biopsy of the tissue. Studies have been done were tissue has been taken from different subjects at different time points but to account for the variation between individuals the population size must be increased. To truly gain an understanding of the change in the concentration of the cytokines over time, a continuous sampling technique is needed.

### 1.2.3 Role in Disease

Traumatic brain injury is a major health concern even with advancements of modern medicine.[22] It is known that the wound healing response within the brain can cause additional damage to tissue around the initial wound. There are currently limited treatments to reduce this additional damage. Since the cytokine network is responsible for the regulation of the wound healing response, investigation into this network within the brain could lead to treatments that would prevent this secondary damage and lead to better outcomes. Cytokines have also been

described in multiple disease states with in the brain.[23-25] While cytokines have been identified with these disease states, their affects and effects on the states are widely not known. The difficulty of understanding their role in the wound response and these diseases is that real time measurements are needed since many cytokines function through a concentration gradient to see if there is a change in this concentration. Real time sampling would allow for experiments to help determine factors that may change these concentrations. In addition there is not a non-invasive way to monitor concentration changes of cytokines with in the brain. Thus a base expectation of cytokine profiles and the expected concentrations caused the sampling procedure must be established before treatments can be compared.

### 1.3 Microdialysis

As stated earlier microdialysis is a sampling technique. This technique is minimally invasive and allows for sampling across time points within the same tissue. The method of microdialysis collection is a perfusate fluid is pumped through a canula. The end of this canula is surrounded by a semi-permeable membrane. Different semi-permeable membranes with different materials chemistry and molecular weight cutoff are available to use for different experiments. A perfusion fluid flows through the membrane inner lumen to be collected. This method of collection is graphically depicted in Figure 1.1.

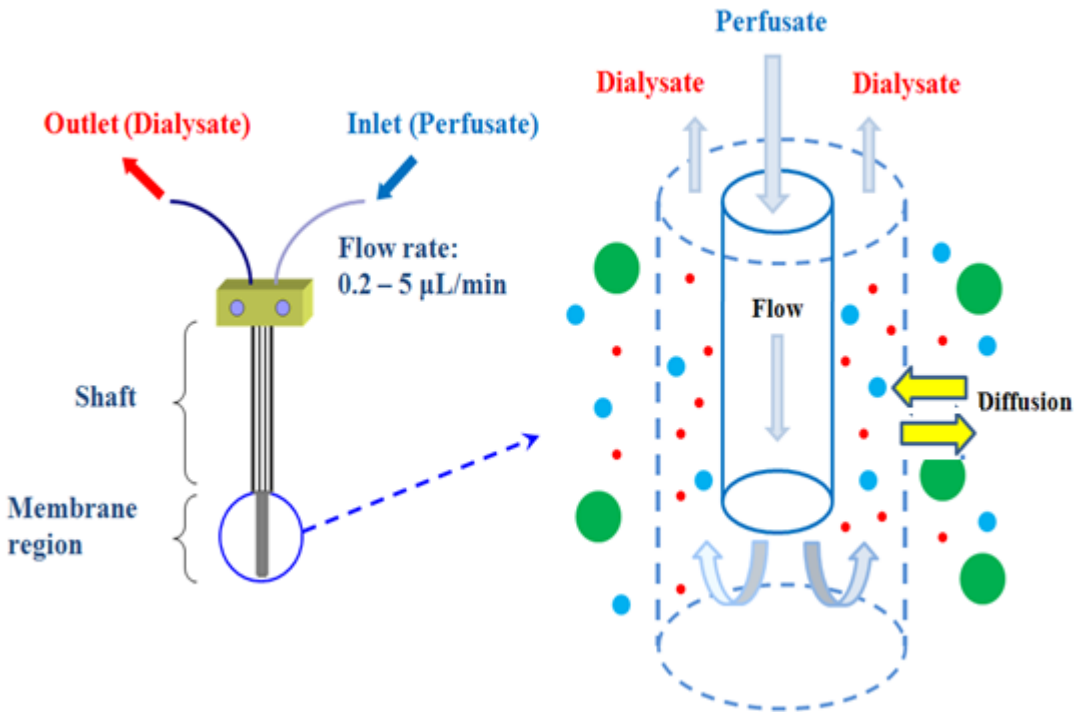



Figure 1.1: Graphical Representation of Microdialysis.[26]

Microdialysis depends on solute passive diffusion for the collection. The factors that control this overall mass transport process include the relation of mass transport resistances of the system. These resistances are described as Resistance to dialysate ($R_d$), Resistance to Membrane ($R_m$), Resistance to Tissue ($R_{ts}$) or in the case of in vitro, Resistance to Sample Media ($R_{sm}$). These resistance values define how easily the analyte moves by diffusion through the areas that they represent. The relation to these resistances and their effect on the Relative Recovery (RR) is shown in Equation 2.

$$RR(\%) = \frac{C_{inlet} - C_{outlet}}{C_{inlet} - C_{Sample,\infty}} \times 100 \qquad (1)$$

Resistance terms are defined in equation 3, 4, and 5. With all terms and symbols defined in the List of Abbreviations and Symbols. In these equations, $r_i$ is the inner membrane radius, $r_\alpha$ is the probe inner cannula radius, $r_o$ is the outer membrane radius, $L$ is the effective membrane length, $D_d$ is the analyte dialysate diffusion coefficient, $D_m$ is the analyte diffusion coefficient through the membrane, $D_q$ is the analyte diffusion coefficient in the quiescent medium external to the probe, $\Phi_m$ is the volume fraction of the membrane accessible to water, and $\Phi_q$ is the volume fraction in the quiescent sample medium.

$$RR = 1 - e^{\left(\frac{-1}{Q_d(R_d+R_m+R_{ts})}\right)} \quad (2)$$

$$R_d = \frac{13(r_i - r_\alpha)}{70\pi L r_i D_d} \quad (3)$$

$$R_m = \frac{ln(r_o/r_i)}{2\pi L D_m \Phi_m} \quad (4)$$

$$R_{ts} = \frac{1}{2\pi D_q \Phi_q \sqrt{2r_o L}} \quad (5)$$

Relative Recovery should not be confused with Absolute Recovery. Absolute Recovery expresses the amount of mass that is collected, because of this it can never go above 100% and can never be negative. Relative Recovery is a comparison of concentrations. The amount of analyte collected is often expressed as relative recovery (RR) or extraction efficiency (EE).

Another benefit to microdialysis is that it allows for the real time analysis of these concentrations. Since the sampling technique is continuous, changes in concentration can be monitored over time. The limitation to this real time analysis is the volume of sample needed for quantification and flow rate used in collection. The sample volume needed is dependent on the detection method used, with a range of 25 µL to 100 µL or more. This means that for lower

volumetric perfusion rates (0.2 -0.5 µL/min) time resolution is limited. Higher volumetric perfusion rates (1-5µL/min) are needed to have better time resolution. The relationship between flow rate and Relative Recovery with known and estimated values for has been described and is shown in Figure 1.2.

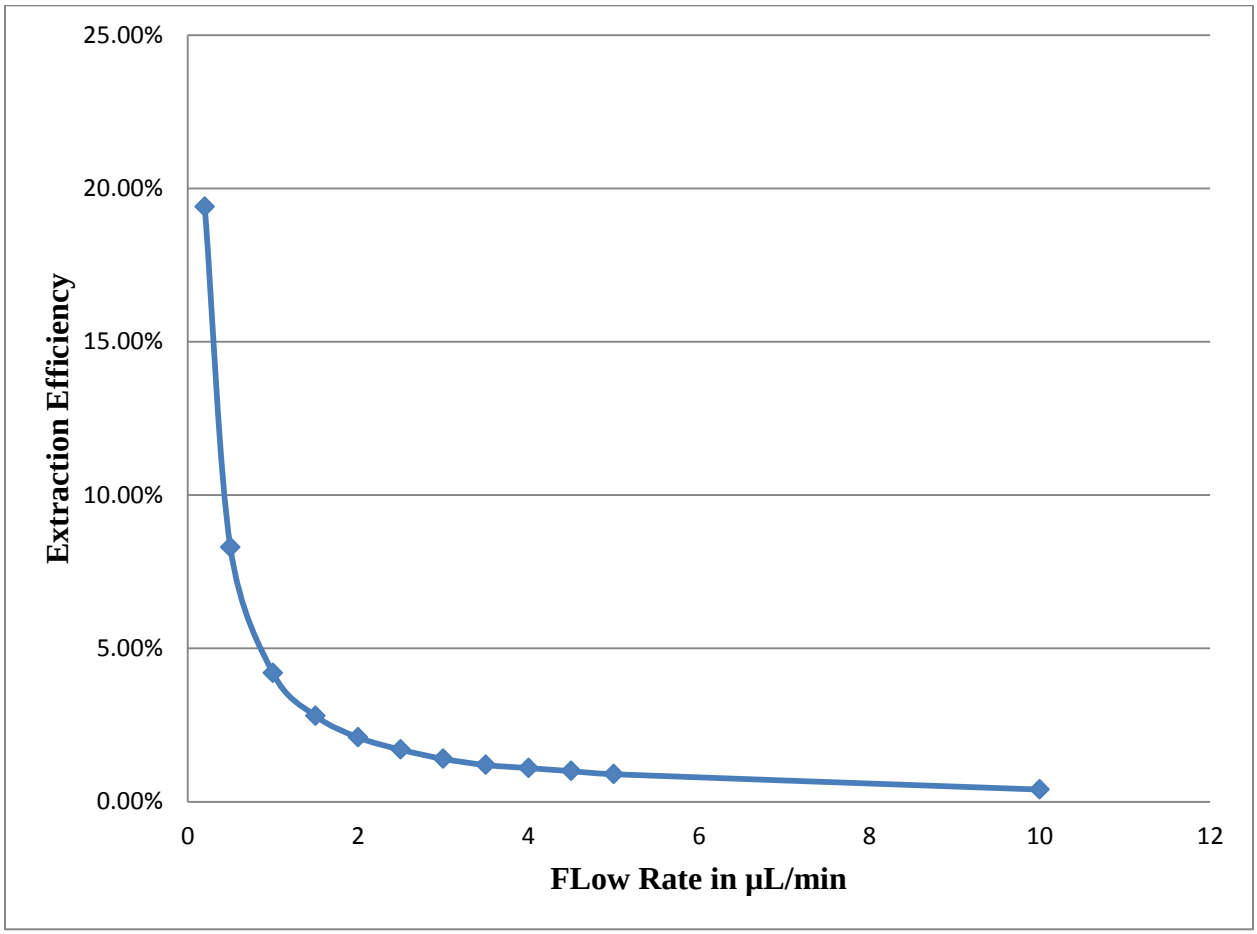



Figure 1.2: Relationship between Flow Rate and Extraction Efficiency

A slower flow allows for a higher relative recovery but also increases the time need to collect the sample. Thus a tradeoff is often needed with respect to time resolution and the amount recovered. Time resolution is the amount of samples that can be taken per increment of time thus

the more samples that can be taken in smaller amounts of time the better the time resolution. It is important to understand when doing microdialysis experiments what time resolution is required and the optimal flow rate that will gather at least the minimal amount of sample volume need in that time frame.

### 1.4 Protein Collection

Microdialysis collection has had some problems with the collection of larger molecules such as proteins. Membranes have been designed with 3000 kDa molecular weight cutoff (MWCO) however for some proteins their recovery still remains low.[26] This poses a particular problem with cytokines as the relative recovery of some cytokines has been reported to be low (10-15%). Waelgaard *et al*. found that the relative recovery of cytokines varies greatly and appears to be dependent on other factors besides size.[27] The exact reasons for these low recovery values are not known at this time, however it is believed that hydrophobic interactions with the membrane is one of the causes. The combination of the low recovery rate and low concentration in vivo systems pushes the limits of current detection methods. In this work we attempt to improve the relative recovery to allow better quantification of cytokines.

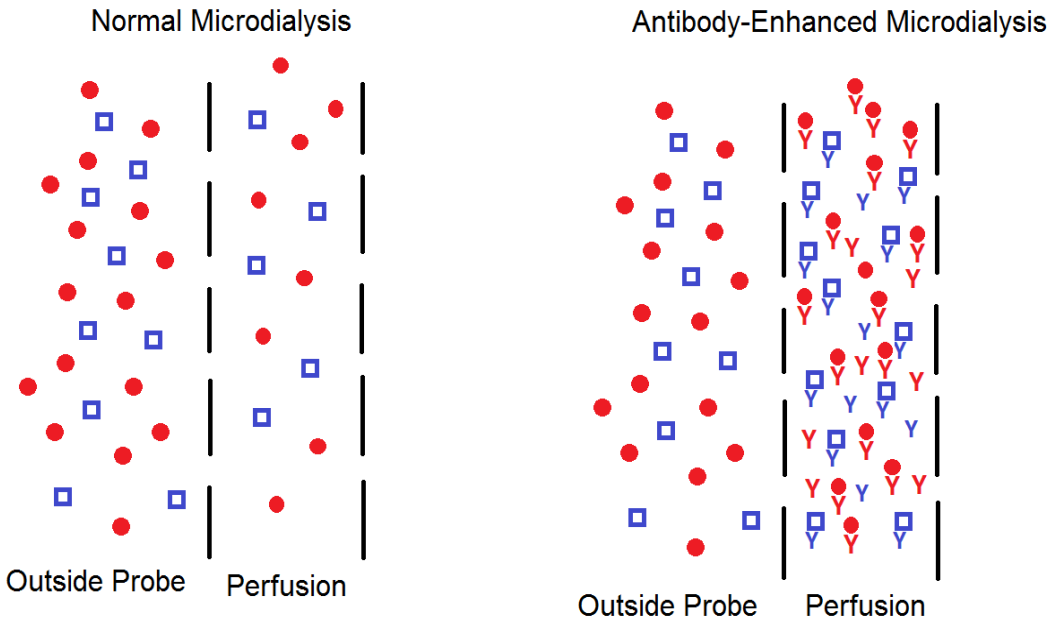



Figure 1.3: Graphical representation of Microdialysis and Antibody-Enhanced Microdialysis.

In this work we use the addition of an affinity agent to the perfusion fluid to increase the net flux across the membrane. This is graphically represented in Figure 1.3. The mechanism of this increase in net flux comes from the reduction of the free analyte in the perfusion fluid by binding to the affinity agent. The reduction in free analyte in the perfusion fluid drives more targeted solute to diffuse across the membrane thus increasing the net flux. Work has been done to identify appropriate affinity agents for cytokines, antibodies and heparin,[28] and human albumin serum.[29] In these experiments we use antibodies as the affinity agents Antibodies have a high specificity for their target as well as a high binding affinity. In this work we will use free antibodies instead of antibodies attached to bead because it has been shown that there is a loss of bead do to aggregation and settling.[28] These values are different for each antibody and antigen but the range of these binding affinities are reported to have $K_{(association)}$ values of approximately

$10^8$. This insures that almost the entire antigen is bound to the antibody if sufficient concentrations of antibody are present. This works well for the affinity agent microdialysis to ensure an increase in flux since there is very little free antigen present in the dialysate. In addition to antibodies, heparin has been used as an affinity agent. While heparin has a lower binding affinity to cytokines than antibodies it has the advantage that after collection the cytokines could be dissociated from it. This could allow for preconcentration of the cytokines before detection.

### 1.5 Enzyme-linked Immunosorbent Assay

One method that is able to quantify within the range of the collected cytokines is Enzyme-linked Immunosorbent Assay (ELISA). ELISA is a specific type of sandwich immunoassay. In a standard sandwich immunoassay a capture antibody is fixed onto a surface, sample is then added and the antigen is allowed to bind to the capture antibody, a second antibody is then added with a marker that is then detected. An ELISA instead of having a marker attached to the second antibody, also known as the detection antibody, an enzyme is attached. Most commonly this enzyme has products that are able to be detected by UV/Vis absorption. Thus once the detection antibody has bound the antigen substrate to the enzyme is added and converted into a product that is then detected. This method allows for amplification of the signal over a standard immunoassay. A graphical representation of the ELISA procedure is depicted in Figure 1.4. ELISA is commonly used to measure cytokines in serum and homogenized tissue [30,31]

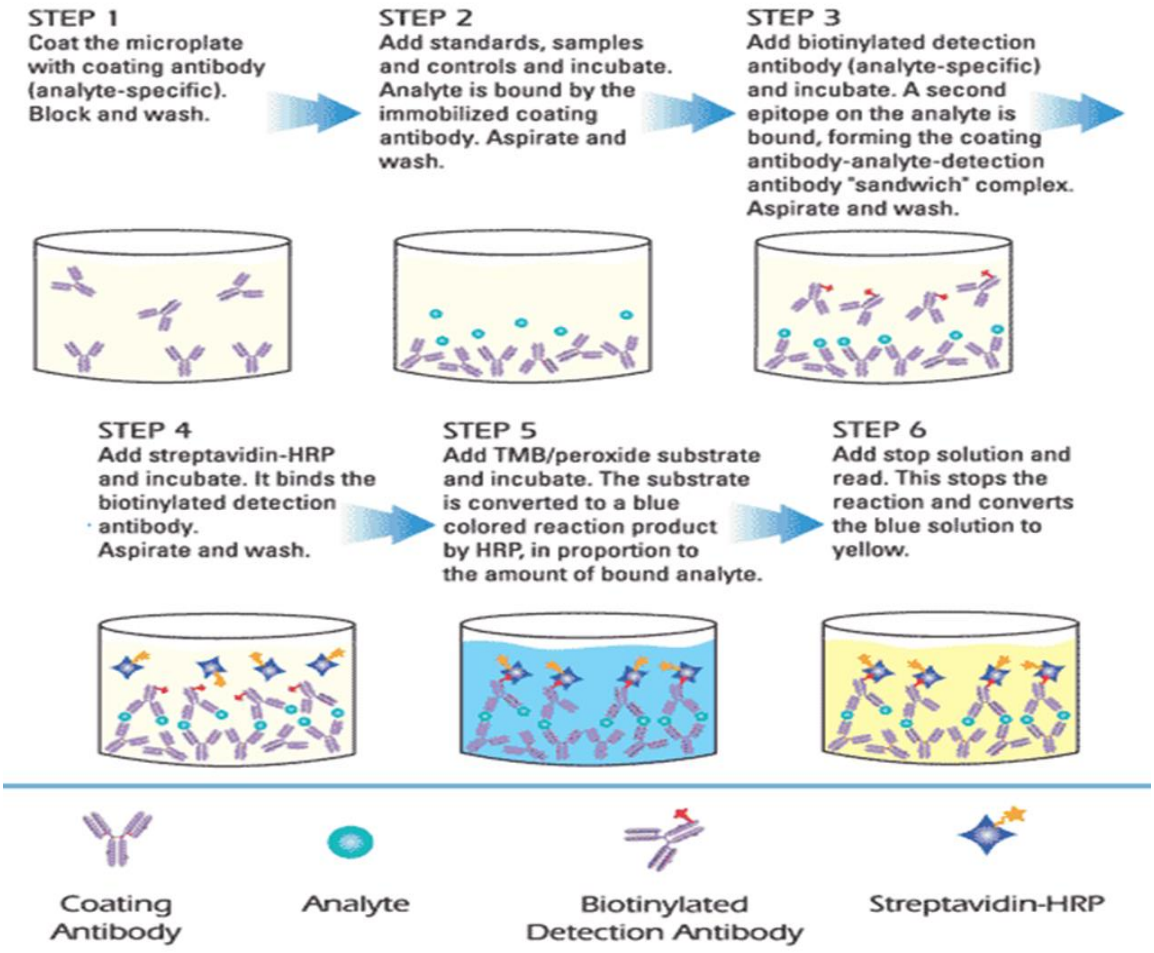
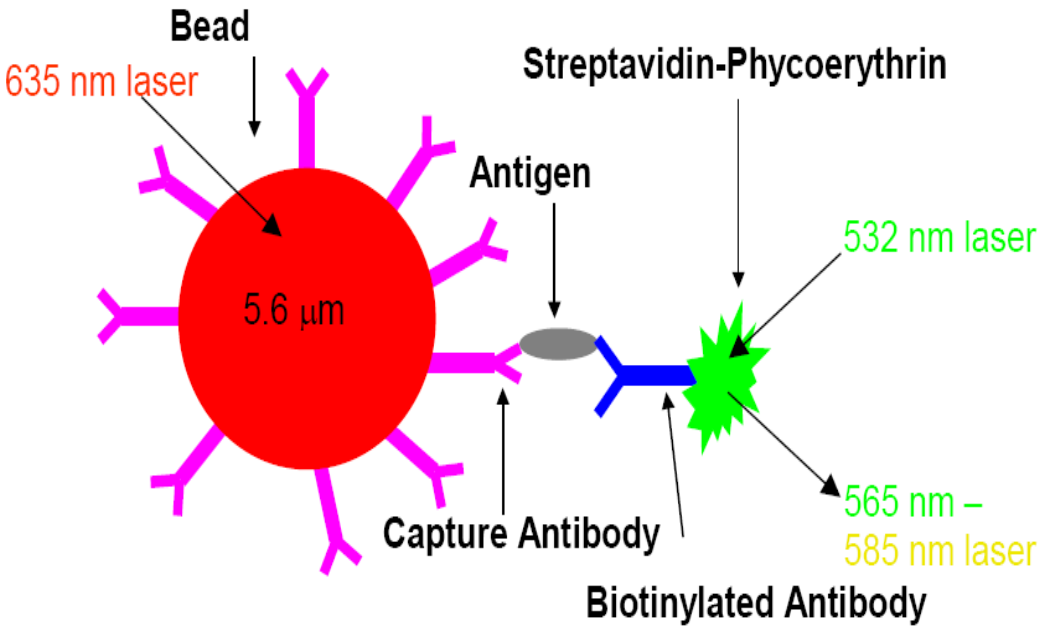



Figure 1.4: Pictorial representation of the ELISA procedure.[32]




**1.6 Bead Based and Microarray Immunoassays**

To reduce the sample volume needed to measure cytokines, development of microarrays and bead based assays have been created. Current commercially available bead based assays require sample volumes as low as 25µL, while developing microarrays can use as little as 20µL.[33-35] Bead based assays are able to reduce the volume needed by increasing the surface area of the assay. In a standard immunoassay the capture antibody is fixed to a flat surface. In a bead based assay the capture antibody is fixed to functionalized beads. The use of these beads increases the amount of surface area and thus the amount of capture antibody. These beads are also suspended in the sample solution thus a smaller overall volume of sample is needed to have the same amount of exposure to capture antibody. Figure 1.5 is a graphical representation of the components of a bead based assay.

Figure 1.5: Pictorial representation of the bead-based assay.[36]

A second advantage to the bead based and microarray assays are that they may be multiplexed. With the same 25µL sample a bead based assay could detect up to 100 different analytes.[34] Developing microarrays also are able to increase the number of analytes detected in a single sample, though the number of analytes solely depends on the fabrication method. One problem with this high level of multiplexing is cross reactivity. Each analyte that you add to the array or bead set must be checked for cross reactivity with the others. This extensive pretesting with this level of multiplexing is time consuming. The benefits of lower sample size needed and multiplexing makes bead based and microarray immunoassays appropriate for detection of several cytokines at once that have been collected by microdialysis. This multiplexing is important since cytokine responses are often described as a network of chemical messengers allowing the researcher to investigate the whole network rather than one cytokine at a time.

### 1.7 Significance and Goal of Thesis

The work presented here attempts to develop microdialysis methods that will increase relative recovery of cytokines which would allow for quantification in later treatment experiments. This work will focus on the use of microdiaylsis as a method to collect cytokines from extracellular fluid in brain tissue. Microdialysis will be used in conjunction with appropriate detection methods, which in this research will focus on ELISA and bead based immunoassay. This collection and quantification will establish extracellular concentrations of cytokines and the changes there in over time. The first part of this work will focus on the increase of relative recovery of cytokines which are in naturally low concentrations in the extracellular fluid. Previous studies of cytokine collections by microdialysis have shown that

they have a low(5% - 20%) relative recovery.[28] This presents a difficulty in quantification of cytokines when microdialysis is used as the sampling method. Cytokines are present in low (pM) range *in vivo.* A low relative recovery means that the concentration of the cytokine in the sample may be below the limits of detection for current quantification methods. The second part of this work will be to test the developed methods *in vivo* to ensure that cytokine relative recovery is increased *in vivo* for use with later treatment experiments.

In Chapter 2 the use of a microdialysis technique of stopped-flow will be discussed for the increase of relative recovery of the cytokines CCL2 and Interferon Gamma (IFN-γ). In the following chapters (Chapters 3) the use of an affinity agent of antibody specific cytokines CCL2 and Interleukin-6 (IL-6) to increase relative recovery both in vitro and in vivo (Sprague-Dawley rats). In the following chapter (Chapter 4) the development of a bead based immunoassay for the cytokine CCL2 will be discussed and compared to commercially available assays.

## 1.8 References


1. Adams, R. N., Probing brain chemistry with electroanalytical techniques. *Analytical Chemistry* 1976, 48, 1126A-1138A.

2. Ungerstedt U Fau - Pycock, C.; Pycock, C., Functional correlates of dopamine neurotransmission.

3. Myers, R. D., An improved push-pull cannula system for perfusing an isolated region of the brain. *Physiology & Behavior* 1967, 5, 243-246.

4. Perry, M.; Li, Q.; Kennedy, R. T., Review of recent advances in analytical techniques for the determination of neurotransmitters. *Anal. Chim. Acta* 2009, 653, 1-22.

5. Levine, J. E.; Ramirez, V. D., Measurement of neuropeptide release: in vitro and in vivo procedures. *Methods Enzymol.* 1986, 124, 466-94.

6. Kottegoda, S.; Shaik, I.; Shippy, S. A., Demonstration of low flow push-pull perfusion. *J Neurosci Methods* 2002, 121, 93-101.

7. Myers, R. D.; Adell, A.; Lankford, M. F., Simultaneous comparison of cerebral dialysis and push-pull perfusion in the brain of rats: a critical review. *Neurosci Biobehav Rev* 1998, 22, 371-87.

8. German, I.; Roper, M. G.; Kalra, S. P.; Rhinehart, E.; Kennedy, R. T., Capillary liquid chromatography of multiple peptides with on-line capillary electrophoresis immunoassay detection. *Electrophoresis* 2001, 22, 3659-3667.

9. Gouwy, M.; Struyf, S.; Proost, P.; Van Damme, J., Synergy in cytokine and chemokine networks amplifies the inflammatory response. *Cytokine & Growth Factor Reviews* 2005, 16, 561-580.

10. Haddad, J. J., Cytokines and related receptor-mediated signaling pathways. *Biochemical and Biophysical Research Communications* 2002, 297, 700-713.

11. Fitzgerald, K. A.; O'Neill, L. A. J.; Gearing, A. J. H.; Callard, R. E., *The Cytokine FactsBook*. 2nd ed.; Academic Press: New York, 2001.

12. Gaudet, S.; Janes, K. A.; Albeck, J. G.; Pace, E. A.; Lauffenburger, D. A.; Sorger, P. K., A Compendium of Signals and Responses Triggered by Prodeath and Prosurvival Cytokines. *Molecular & Cellular Proteomics* 2005, 4, 1569-1590.

13. Chui, R.; Dorovini-Zis, K., Regulation of CCL2 and CCL3 expression in human brain endothelial cells by cytokines and lipopolysaccharide. *Journal of Neuroinflammation* 2010, 7.

14. Schilling, M.; Strecker, J. K.; Schabitz, W. R.; Ringelstien, E. B.; Kiefer, R., Effects of Monocyte Chemoattractant Protein 1 on Blood-Borne Cell Recruitment After Transient Focal Cerebral Ischemia in Mice. *Neuroscience* 2009, 161, 806-812.

15. Magge, S. N.; Malik, S. Z.; Royo, N. C.; Chen, H. I.; Yu, L.; Snyder, E. Y.; O'Rourke, D. M.; Watson, D. J., Role of Monocyte Chemoattractant Protein-1 (MCP-1/CCL2) in Migrationof Neural Progenitor Cells Toward Glial Tumors. *Journal of Neuroscience Research* 2009, 87, 1547-1555.

16. Cho, C.; Miller, R. J., Chemokine receptors and neural function. *J. NeuroVirol.* 2002, 8, 573-584.

17. Adler, M. W.; Geller, E. B.; Chen, X.; Rogers, T. J., Viewing chemokines as a third major system of communication in the brain. *AAPS J.* 2005, 7, E865-E870.

18. Gabellec, M.-M.; Griffais, R.; Fillion, G.; Haour, F., Interleukin-1 receptors type I and type II in the mouse brain: kinetics of mRNA expressions after peripheral administration of bacterial lipopolysaccharide. *J. Neuroimmunol.* 1996, 66, 65-70.

19. Ignatowski, T. A.; Noble, B. K.; Wright, J. R.; Gorfien, J. L.; Heffner, R. R.; Spengler, R. N., Neuronal-associated tumor necrosis factor (TNFα): its role in noradrenergic functioning and modification of its expression following antidepressant drug administration. *J. Neuroimmunol.* 1997, 79, 84-90.

20. Agnati, L. F.; Guidolin, D.; Guescini, M.; Genedani, S.; Fuxe, K., Understanding wiring and volume transmission. *Brain Research Reviews* 2010, 64, 137-159.

21. Ambrosini, E.; Aloisi, F., Chemokines and Glial Cells: A Complex Network in the Central Nervous System. *Neurochemical Research* 2004, 29, 1017-1038.

22. Ghirnikar, R. S.; Lee, Y. L.; Eng, L. F., Inflammation in traumatic brain injury: role of cytokines and chemokines. *Neurochem. Res.* 1998, 23, 329-340.

23. Bauer, S.; Cepok, S.; Todorova-Rudolph, A.; Nowak, M.; Koeller, M.; Lorenz, R.; Oertel, W. H.; Rosenow, F.; Hemmer, B.; Hamer, H. M., Etiology and site of temporal lobe epilepsy influence postictal cytokine release. *Epilepsy Res.* 2009, 86, 82-88.

24. Conductier, G.; Blondeau, N.; Guyon, A.; Nahon, J.-L.; Rovère, C., The role of monocyte chemoattractant protein MCP1/CCL2 in neuroinflammatory diseases. *Journal of Neuroimmunology* 2010, 224, 93-100.

25. Dunn, A. J. In *Effects of cytokines and infections on brain neurochemistry*, Academic Press: 2001; pp 649-666.

26. Ao, X.; Stenken, J. A., Microdialysis sampling of cytokines. *Methods (San Diego, CA, U. S.)* 2006, 38, 331-341.

27. Waelgaard, L.; Pharo, A.; Toennessen, T. I.; Mollnes, T. E., Microdialysis for monitoring inflammation: efficient recovery of cytokines and anaphylotoxins provided optimal catheter pore size and fluid velocity conditions. *Scand. J. Immunol.* 2006, 64, 345-352.

28. Duo, J. Affinity Microdialysis Sampling of Cytokines. Rensselaer Polytechnic Institute, Troy, New York, 2009.

29. Helmy, A.; Carpenter, K. L. H.; Skepper, J. N.; Kirkpatrick, P. J.; Pickard, J. D.; Hutchinson, P. J., Microdialysis of cytokines: methodological considerations, scanning electron microscopy, and determination of relative recovery. *J Neurotrauma* 2009, 26, 549-61.

30. Thompson, W.; Karpus, W.; Van Eldik, L., MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *Journal of Neuroinflammation* 2008, 5, 35.

31. Kreuzer, K.-A.; Rockstroh, J. K.; Sauerbruch, T.; Spengler, U., A comparative study of different enzyme immunosorbent assays for human tumor necrosis factor-α. *J. Immunol. Methods* 1996, 195, 49-54.

32. Thermoscientific Pictorial representation of the ELISA procedure. http://www.thermoscientific.com/wps/portal/ts/products/catalog?categoryId=92512.

33. Yuan, J., Cytoplex bead-based technology for clinical diagnosis. *J. Proteomics Bioinf.* 2009, S3, No pp. given.

34. Hsu, H.-Y.; Joos, T. O.; Koga, H., Multiplex microsphere-based flow cytometric platforms for protein analysis and their application in clinical proteomics - from assays to results. *Electrophoresis* 2009, 30, 4008-4019.

35. Gonzalez, R. M.; Seurynck-Servoss, S. L.; Crowley, S. A.; Brown, M.; Omenn, G. S.; Hayes, D. F.; Zangar, R. C., Development and Validation of Sandwich ELISA Microarrays with Minimal Assay Interference. *J. Proteome Res.* 2008, 7, 2406-2414.

36. Fletcher, H. J.; Stenken, J. A., An in vitro comparison of microdialysis relative recovery of Met- and Leu-enkephalin using cyclodextrins and antibodies as affinity agents. *Anal. Chim. Acta* 2008, 620, 170-175.

## 2.0 Stopped Flow Microdialysis of Chemokine(C-C motif) Ligand 2(CCL2/ MCP-1) and Interferon-gamma (IFN-γ)

### 2.1 Introduction

In this chapter, experiments using stopped flow microdialysis to attempt an improvement in the relative recovery of cytokines are described. Stopped flow microdialysis is a method of microdialysis sampling in which the perfusion fluid is stopped for a certain amount of time and then the flow is resumed. Stopped flow microdialysis is often used in delivery studies, where the drug of interest is added to the perfusion fluid and allowed to diffuse into the tissue to either suppress or solicit an analyte of interest that is then collected. It is uncertain what factors are responsible for the low level of collection of cytokines through microdialysis. In these experiments we explore this question and attempt to establish a method that allows for a greater collection of cytokines. In these experiments we attempt to give the cytokine the longest residence time possible. It is hypothesized that an increase in residence time will increase the Relative Recovery. To test this hypothesis the stopped flow method was proposed.

In these experiments two cytokines, Chemokine (C-C motif) Ligand 2(CCL2/MCP-1) and Interferon-gamma (IFN-γ), were examined. IFN-γ was selected because it is present at concentrations (5-10 pg/ml)[1] which are at the lower limits of current detection methods as described in chapter 1. CCL2 was selected as a second cytokine because of the wide interest for measuring this cytokine in neurological disorders. An improvement in recovery would lead to a higher concentration in the sample. A higher concentration within the sample would prevent some samples being below the limits of detection of the ELISA. This would allow for a greater

sampling concentration range with-in the tissue for areas of interest that may have lower *in vivo* concentrations.

## 2.2 Experimental Section

### 2.2.1 Materials

Rat MCP-1 and rat IFN-γ was purchased from Prepro-Tech INC (Rocky Hill, NJ). Microdialysis probes CMA/20 14/10 polyethersulfone membrane (PES) and 10-mm length and CMA 402 Syringe Pump were purchased from CMA/Microdialysis (Stockholm, Sweden). Fetal bovine serum (FBS) was purchased from Sigma (St. Louis, MO). Rat MCP-1 BD optEIA ELISA kit and rat BD optEIA IFN-γ kit were purchased from BD Biosciences (San Jose, CA). Absorbance was measured with Infinite M200 plate reader purchased from Tecan (Research Triangle Park, NC).

### 2.2.2 Methods

#### 2.2.2.1 Stopped Flow

Sample solutions with concentrations of 2000 pg/mL of CCL2 or IFN-γ in phosphate buffered saline (PBS) with 10% fetal bovine serum (FBS) by volume at pH 7 were generated. Cytokine samples were then placed on a heated stir plate (35 ºC) and mixed during microdialysis sampling. A perfusion fluid of PBS with 10% FBS was then made. Microdialysis probes were inserted into the sample and a flush was performed at 5µL/min until all air was removed from the tubing. Two stopped flow times were used in separate experiments: one for 30 minutes and the

second for 60 minutes. Following the stopped flow, two collection flow rates were tested: one of 5µL/min and one of 2 µL/min. Samples were collected for 15 minutes, which delivered a sample volume of 75 µL and 30 µL, respectively. For the 2 µL/min the process was then repeated to give a final sample volume of 60 µL. Samples were frozen and stored at -20°C for no longer than 48 hours prior to analysis.

Once thawed, 50 µL was taken from each and mixed with 50 µL of the ELISA assay diluent, which brought the final sample volume to 100 µL as per kit specifications. These samples were then analyzed according to kit protocols and the absorbance was measured by an Infinite M200 plate reader. Quantification was performed by a comparison to standards on the same plate.

#### 2.2.2.2 Continuous flow

Sample solutions with concentrations of 2000 pg/mL of CCL2 or IFN-γ in PBS with 10% FBS at pH 7 were used. These solutions were then placed on a heated stir plate (35 ºC) and mixed while sampling. A perfusion fluid of PBS with 10% FBS was then made. Microdialysis probes were inserted into the sample and a flush was performed at 5µL/min until all air was removed from the tubing. Flow rates of 0.5, 1, and 2µL/min were then used to sample for 60 minutes, giving sample sizes of 30, 60 and 120 µL, respectively. Samples were then frozen and stored for no longer than48 hours at -20°C. Once thawed, sample volumes of 30, 50, and 100 µL were taken and mixed with 70, 50, and 0 µL assay diluent, respectively, to bring the final sample volume to 100 µL. The samples were then run according to kit protocols and the absorbance was measured by an Infinite M200 plate reader. Quantification was performed by a comparison to a standard curve run on the same plate.

### 2.2.2.3 Methyl Orange

A solution of 10 µM Methyl Orange in 10mM phosphate buffer was generated. It was heated to 35°C and stirred during collection. A perfusion fluid of 10mM phosphate buffer was flushed and allowed to sit for 30 minutes or 1 hour in two trials. A collection flow rate of 2 µL/min was used to collect for 15 minutes to give a volume of 30 µL, this was repeated after a second stopped flow to allow for a final sample volume of 60 µL. Three samples of 60 µL for each stopped time were then compared to a continuous flow of 2 µL/min. This was done to duplicate the methods used in the cytokine collection. Absorbance was then measured using Infinite M200 plate reader.

### *2.2.3* Work-Flow Diagram

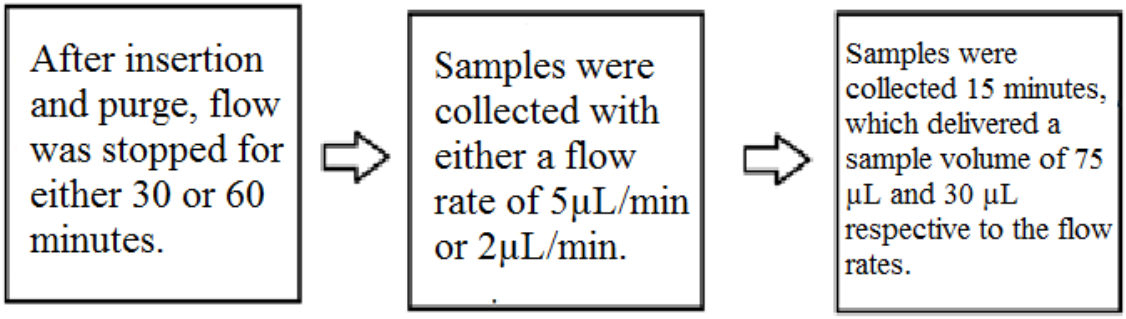



Figure 2.1 Work Flow diagram of Stopped-Flow Experiments.

### 2.2.2.3 Statistics

Stopped Flow data were compared to each other with respect to stopped flow times and recovery flow rates using ANOVA (Microsoft Excel). Stopped Flow data were compared to Continuous Flow data using ANOVA(Microsoft Excel).

## 2.3 Results

### 2.3.1 Stopped Flow Collections of CCL2

It was found that the stopped flow microdialysis gave a very large range of recovery values. In some cases the collection value would be greatly improved, while in others the amount recovered was below the detection limits. Figure 2.2 shows the average of six collections with error bars representing one standard deviation.

The 5 µL/min collection flow rate gave average relative recoveries of 14.79%±14.19% and 12.40%±11.83% at 60 minutes and 30 minutes stopped flow times, respectively. The 2 µL/min flow rate collection gave average relative recoveries of 17.27%±16.80% and 14.51%±14.06% at the 60 minute and 30 minute stopped flow times, respectively. No significant difference was seen with the stopped flow in respect to stopped flow time or collection flow rates ($p=0.21$, $p=0.19$ respectively).

### 2.3.2 Continuous Flow Collections of CCL2

Continuous flow gave average values where $n=6$ of 5.58% ± 2.71%, 3.39% ± 1.32%, and 1.92% ± 0.97% at flow rates of 0.05 µL/min, 1 µL/min, and 2 µL/min, respectively. This is shown graphically in Figure 2.3. No significant difference was seen in the flow rates ($p=0$ .13).

### 2.3.3 Stopped flow Collections of IFN-γ

The stopped flow of the IFN-γ also evidenced large variation. Recorded relative recoveries of 8.95%±8.01% and 7.27%±.14% (n=6) were observed at a collection flow rate of 5 µl/min for 30 minutes and 60 minutes stopped flow, respectively. Relative recoveries of 5.93%±3.07% and 3.95%±3.82% were observed at collection flow rate of 2µL/min at 30 minutes and 60 minutes stopped flow, respectively. This is shown graphically in Figure 2.4. No significant difference was seen in the stopped flow in respect to stopped flow time or collection flow rates( $p = 0.17$ , $p = 0.15$ respectively).

### 2.3.4 Continuous flow Collection of IFN-γ

Continuous flow collection of IFN-γ gave average relative recoveries of 5.57% ± 2.16%, 2.33% ± 1.48%, and 0.81% ± 0.20% at flow rates of 0.5, 1, and 2 µL/min. This is shown graphically in figure 2.5. No significant difference was seen in the flow rates ($p = 0.11$).

## 2.4 Methyl Orange Stopped Flow and Continuous Flow Collection

Continuous flow collection of Methyl Orange gave an average relative recover of 34.23%±2.1%. Stopped flow with a stopped time of 30 minutes gave an average relative recovery of 47.21%±3.41%. Stopped flow with a stopped time of one hour gave an average relative recovery of 49.14%±2.67%. Significant difference was seen between both stopped flow times in comparison to the continuous flow and no significant difference between the two stopped flow times ($p = 0.003$, $p = 0.34$ respectively)

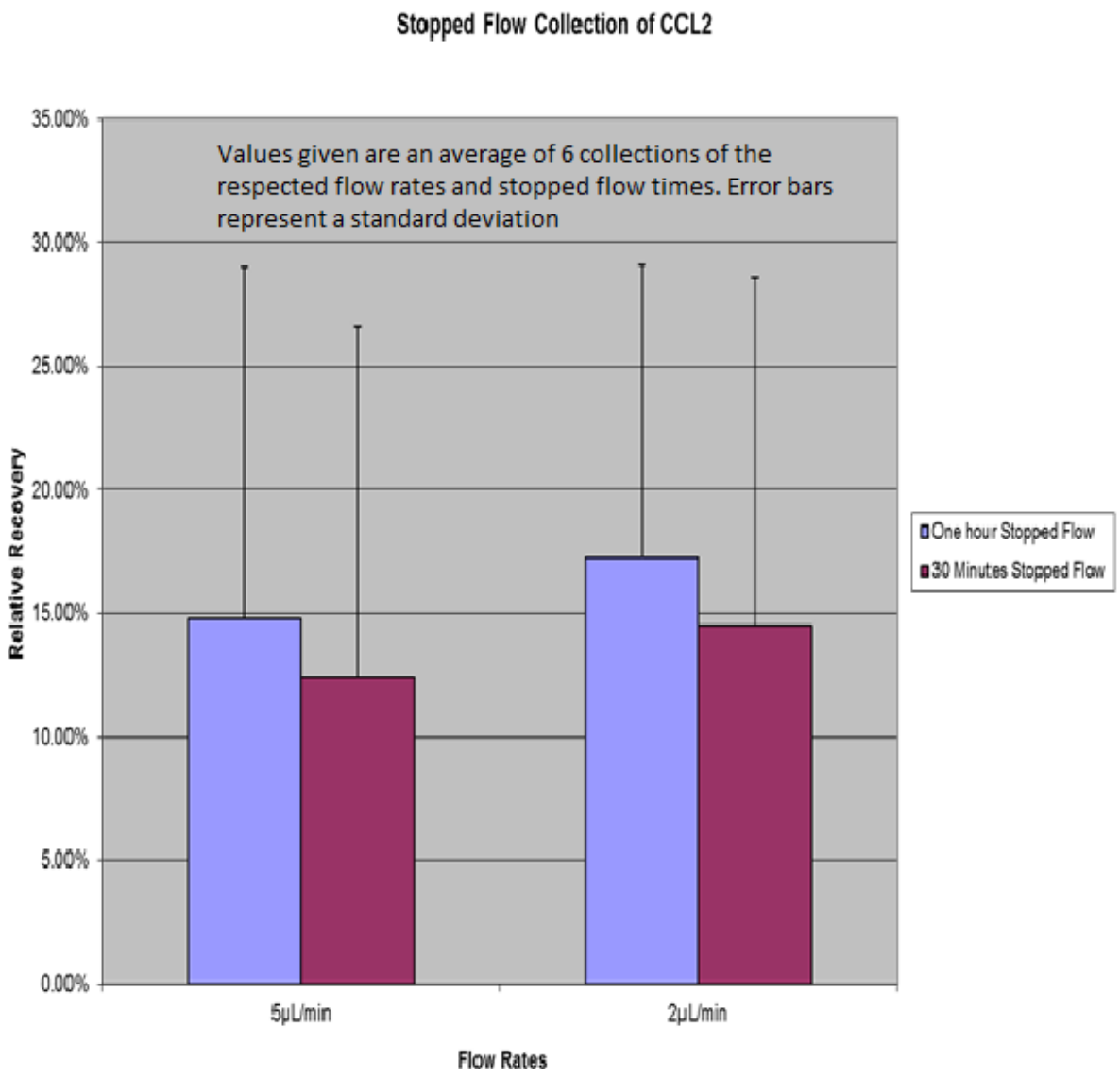



Figure 2.2 Results of Stopped Flow Collection of CCL2.

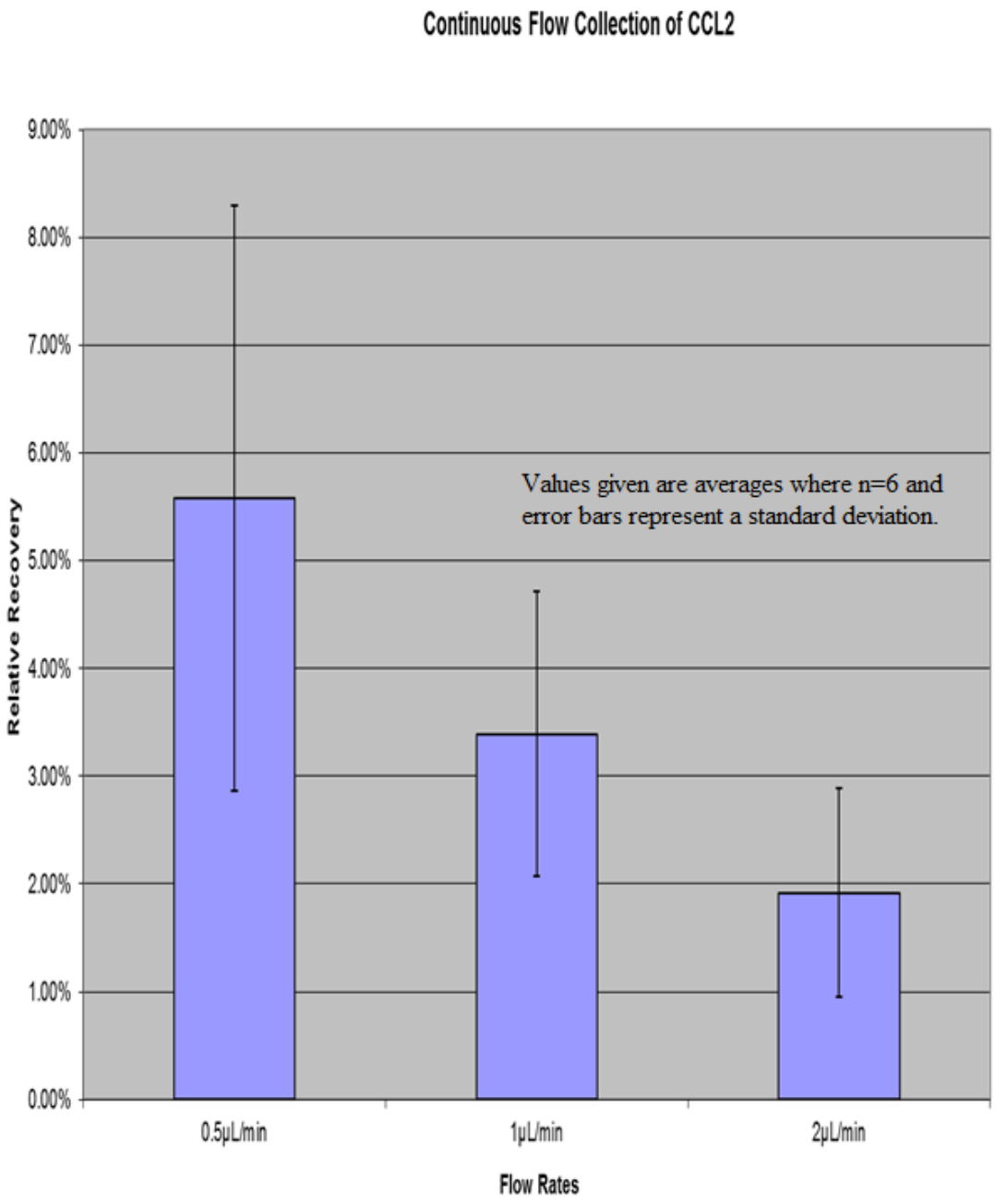



Figure 2.3 Results of Continuous Flow of CCL2.

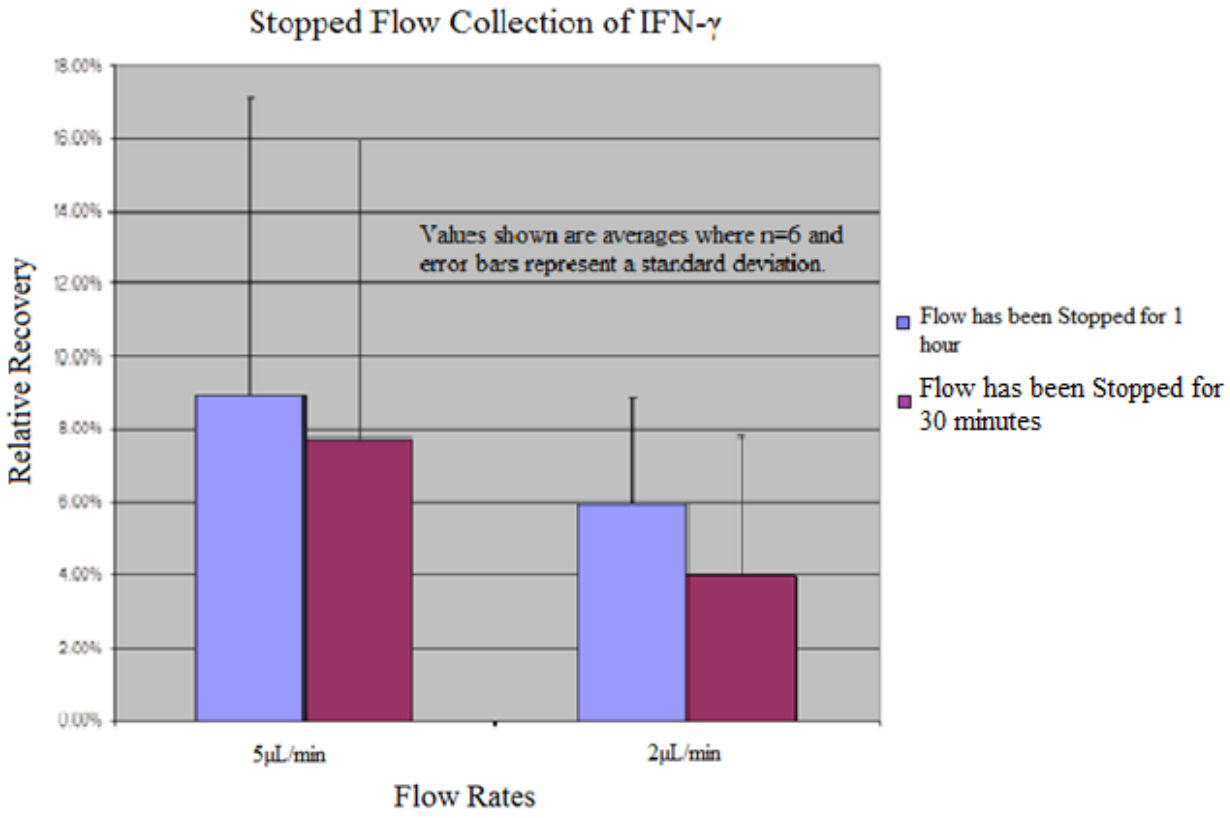



Figure 2.4 Results of Stopped Flow Collection of IFN- γ.

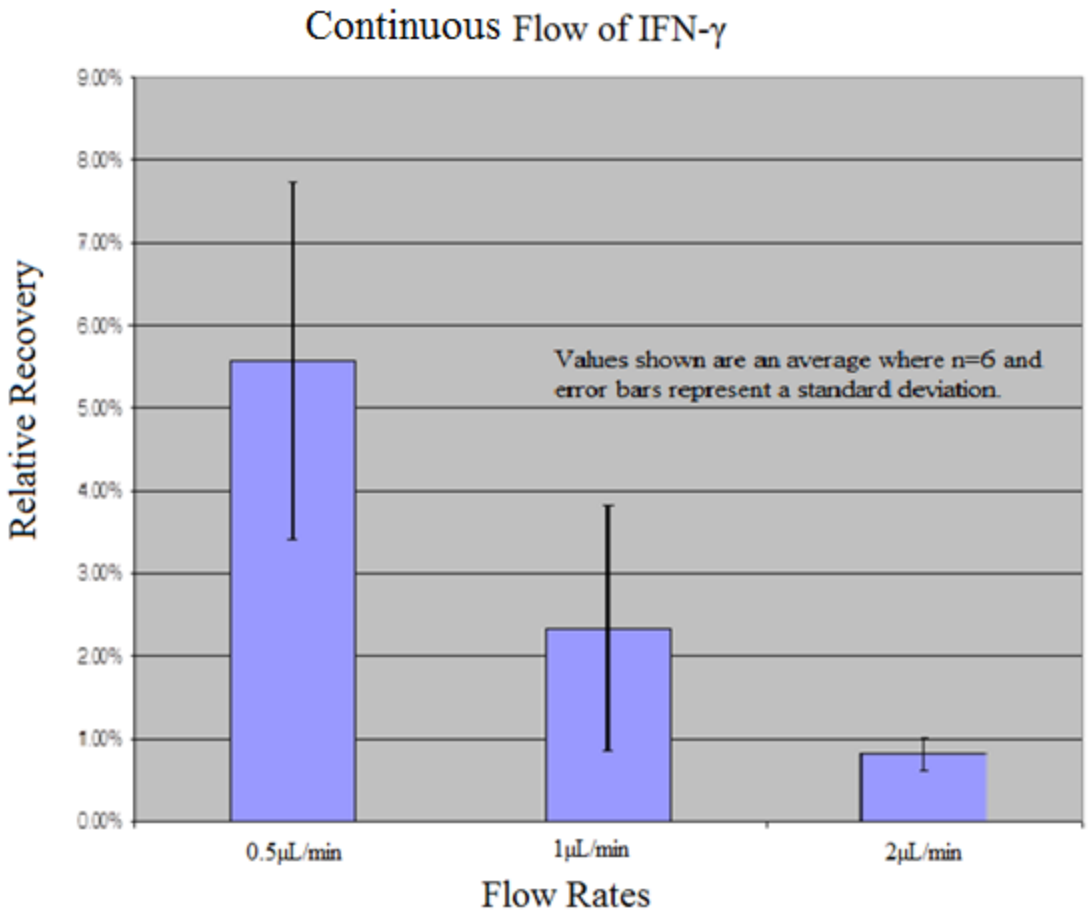



Figure 2.5 Results of Continuous Flow of IFN-γ

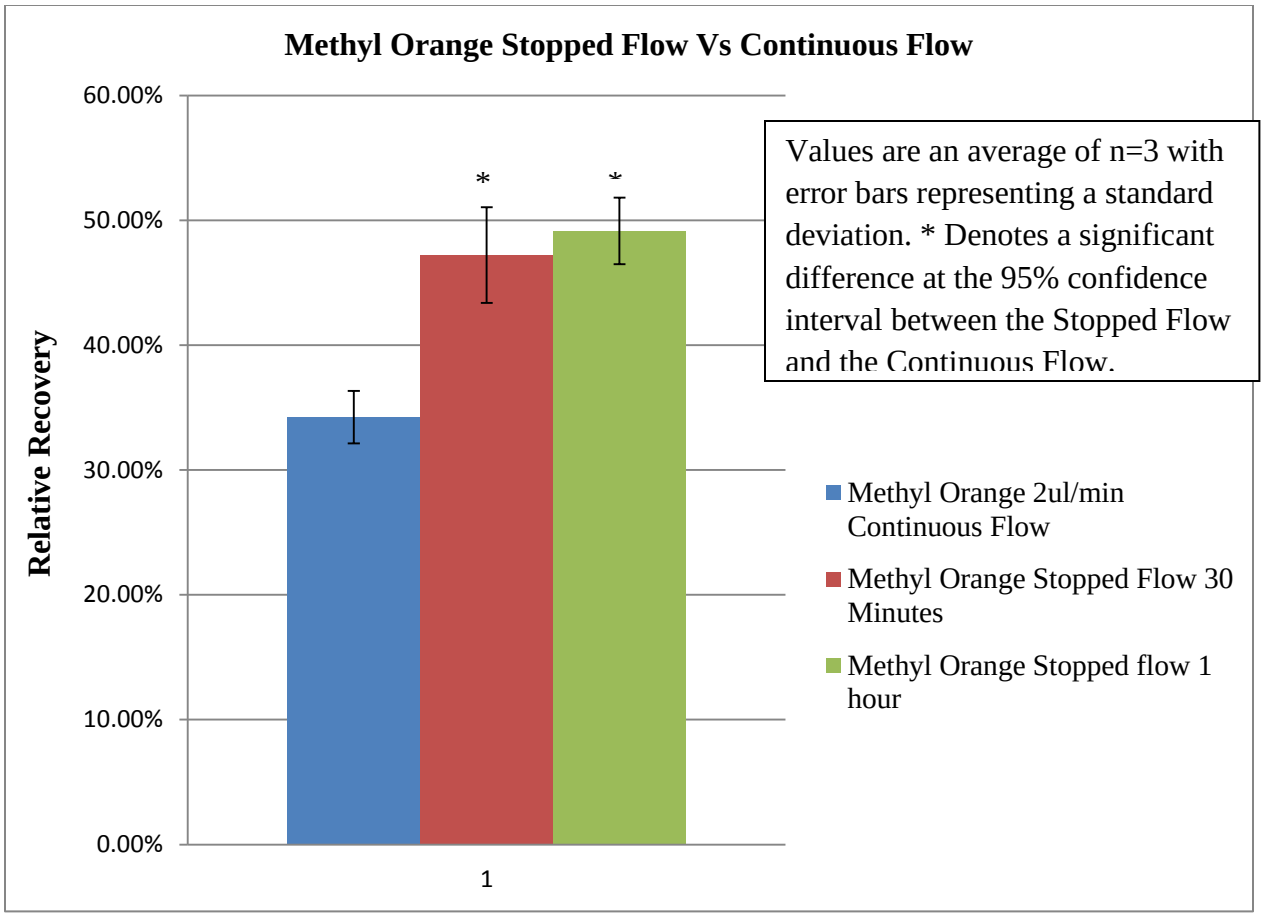



Figure 2.6: Results of Methyl Orange Stopped Flow

**2.4 Discussion**

In these experiments, the use of stopped flow microdialysis to increase the relative recovery of two cytokines, CCL2 and IFN-γ, was investigated. In both studies the stopped flow had an average relative recovery higher than that of the continuous flow, an increase in percentage of 11.69% more recovery in CCL2 and an increase of 3.38% in IFN- γ with respect to their highest percentages from stopped flow to continuous. However, the variation was so great that this could not be stated as a significant increase, or that it was a reliable increase for each sampling. There are several factors that could be causing the large variation in the stopped flow

microdialysis, including but not limited to run to run error, non-specific absorption, analyte loss due to degradation, and immunoassay error.

The relative recovery values 5.6% and 5.5% for CCL2 and INF-γ of the continuous flow experiments are similar to those previously reported of 8.6% and 1.3% respectively.[1] This supports the hypothesis that there may be an effect in the stopped flow sampling that is having an increase effect on the variation. Since the samples were both frozen no longer than 48 hours and quantified by the same technique, it is possible that the extended time in the stopped flow experiments allowed the cytokines to bind to places of lower affinity. In the continuous flow experiments, the cytokines would not have enough time to non-specifically bind to areas of lower affinity. If this is the case, then the non-specific binding amounts would be hard to predict. In addition, with longer sampling time, a saturation effect should be seen. Also, an experiment in which a high concentration of cytokine is infused through the probe before sampling to saturate the non- specific sites may decrease this variation. If the variation caused by the non-specific binding is lowered, the stopped flow method may pose as a valid way of increasing the relative recovery of these cytokines.

This reduction in variation may also allow for the comparison of the 5 µL/min to 2 µL/min collection flow rates. With the current data, there is no significant difference between the two collection flow rates. This is contradictory to established microdialysis theory, since the 2 µL/min should have higher relative recovery than the 5 µL/min. With a reduction in the variation, a difference may be seen and a comparison could be performed. In addition, a comparison of the 30 minute versus the 60 minute stopped flow times could be done. It may be worthwhile to see if the longer time had any impact on the effectiveness of the collection.

In an attempt to further understand this variation an experiment using methyl orange instead of the cytokines was performed. Following the same methods used with the cytokine the variation was able to be reduced to see significant difference between the stopped flow and the continuous flow. Given these results it is currently believed that the variation with the cytokine experiments was most likely caused by the size of the molecule and its tortuous path through the probe under equilibrium

## 2.5 References

1. Duo, J. Affinity Microdialysis Sampling of Cytokines. Rensselaer Polytechnic Institute, Troy, New York, 2009.

## 3.0 In Vivo Collection and Quantification

### 3.1 Introduction

A variety of different experimental approaches have been applied to elucidate the presence of cytokines in the central nervous system. These methods include: 1) mRNA analysis via *in situ* hybridization or Northern blot analysis;[1] 2) radioimmunoassay or ELISA for protein content; 3) radiolabeled cytokine studies in brain slices using autoradiography to map out receptor sites; 4) immunohistochemistry; 5) different imaging methods to map receptors;[2] and 6) different cell lines exposed to different permutations of cytokines to release neuropeptides or exposure of neuropeptides to release cytokines. While all these measurement methods serve their purpose to map out the different cytokine locations, none of these methods allows for real-time in vivo cytokine collection, which is of great interest to numerous neuroscience researchers.

The difficulty with all of these different techniques is that either they do not provide actual protein concentrations especially from the extracellular space or the animal has to be sacrificed to obtain protein content. For example, mRNA expression does not always equate to actual protein concentrations.[3, 4] Immunohistochemical analyses do not allow for measurements of concentrations over time within the same animal. For these reasons, there has been interest in collecting cytokines from the brain using microdialysis sampling. Microdialysis has been used to collect chemokines and cytokines in the brain, but these studies have been predominantly in humans since the length of the membranes is typically 10 mm vs. the 1 to 4 mm range used for rodents. [5-7]

Cytokines have been collected from rat brain under traumatic injury conditions.[8] The difficulty with collecting cytokines is their low basal (pg/mL) concentrations combined with low

recovery through the microdialysis probe. Typically collected cytokines with relative recoveries (RR= $C_{dialysate}/C_{sample}$) between 5 and 10% for 10 mm 100 kDa MWCO membranes.[9] For this reason, we have been using either free antibodies [10] or antibodies immobilized to polymeric beads used in the typical Luminex assay for improvement of protein recovery into microdialysis sampling probes.[11]

In this work, affinity microdialysis was used with a polyclonal (detection) antibody specific for CCL2. This antibody was included in the microdialysis sampling perfusion fluid allowing the collected dialysate to be quantified using a standard ELISA.

### 3.2 Methods

#### 3.2.1 In Vitro

Microdialysis probes (CMA 20 PES, 10 mm length, 100 kDa MWCO, CMA Microdialysis, Stockholm, Sweden) were immersed into a 5 mL solution of 2 ng/mL CCL2 (Preprotech, Rocky Hill, NJ) in 10 mM PBS. The solution was heated to 35ºC and stirred. An aECFperfusion fluid (153.3mM $Na^+$, 4.3mM $K^+$, 0.41 mM $Mg^{2+}$, 0.71 mM $Ca^{2+}$, 139.4 mM $Cl^-$, pH 7.4) with 5% BSA was used as a control. The aECF is an artificial extracellular fluid described by McNay and Sherwin.[12] For antibody-included perfusions the aECF fluid was supplemented with the detection antibody from a BD Opt EIA kit (BD Biosciences, San Diego, CA) to a dilution of 1:500 from the provided material. The protein concentration for the antibody in solution is not provided and is considered proprietary by the manufacturer. Samples were obtained every 30 minutes during a 180 min time period. Samples were stored at -20ºC and were quantified with BD optEIA MCP-1 Kit the following day.

### 3.2.2 In vivo

#### 3.2.2.1 Surgery

Male Sprague-Dawley rats were purchased from Harlan Laboratories, Inc (Madison, WI). Rats were housed in an environmentally-controlled facility with a 12-hour on/off light cycle and *ad libitum* access to food and water. Six rats (275-350 g) were assigned to either a control or antibody-included perfusion regime. Isoflurane (5%) was administered at a flow rate of 0.8 L/min by vaporizer. The rat was secured to the stereotaxic unit and anesthesia was maintained by 1-2% isoflurane at 0.5 - 0.8 L/min as needed. Coordinates of 4.6 mm anterior to bregma, +0.5mm lateral, and +0.5 mm dorsal along the horizontal zero plane (ear bars) were used. After sampling, the probe was removed and the rat was euthanized by $CO_2$. All protocols were approved by the University of Arkansas IACUC.

#### 3.2.2.2 Microdialysis

CMA 12 PES (4 mm, 100 kDa MWCO, CMA Microdialysis, Inc., North Chelmsford, MA) probes were inserted into the brain. The aECF perfusion fluid was used in one set of animals as a control (n=3). The aECF with antibody (BD Opt EIA detection antibody dilution of 1:500) was used in a second set of animals (n=3). After an initial 5 minute flush at 5 µL/min, a flow rate of 2 µL/min was used to collect dialysates, and samples were collected every 30 minutes for 3 hours. Samples were stored on ice, and kept frozen at -20ºC, no longer than three days, until quantified using ELISA. We have previously demonstrated that samples remain stable for at least one week under these conditions.

### 3.2.2 ELISA

Samples were then quantified with BD optEIA Rat MCP-1 ELISA kit (BD Biosciences, San Diego, CA). To meet kit volume specifications, assay diluent (50 µL) from the BD optEIA kit was added to dialysate samples (50 µL) to a final sample volume of 100µL. A comparison of standards that included the 1:500 dilution of detection antibody to control standards was performed. Samples that included the diluted antibody were treated the same way as samples without antibody or standards. This included the required step of adding the detection antibody to all samples during the sample preparation steps involved with the ELISA. The UV absorbance was measured with a Tecan infinite M200 plate reader (Tecan, Research Triangle Park, NC).

### 3.2.4 Statistics.

Antibody-enhanced values were compared to control values at each time point with a paired Students t-test at the 95% confidence level using Microsoft Excel®. ANCOVA was performed using JMP8® software (SAS Institute, Cary, NC).

## 3.3 Results

### 3.3.1 Calibration Curve Statistics

The calibration curves were compared using an Analysis of Covariance (ANCOVA). There was no significant difference found between the slopes of the calibration curves

(normalized to log-log plots) between samples handled using the standard ELISA protocol and antibody-inclusion protocol, p=0.44.

### 3.3.2 In Vitro Microdialysis of CCL2

Antibody enhanced microdialysis in vitro showed a significant increase in the relative recovery of CCL2 vs. control for both the 1.0 and 2.0 µL/min flow rates figure 3.1. The recovery was 37.5% ±10.2% and 64.8% ±11.7% at flow rates of 2.0 µL/min and 1.0 µL/min, respectively. For the controls, the recovery values were 9.6% ±3.4% and 12.2% ±4.1% (n=10), respectively, noting there is not a significant difference in the values. These data are represented in Figure 3.1

### 3.3.3 In Vivo Microdialysis of CCL2

Antibody-included microdialysis samples had CCL2 concentrations of 500±30, 810±100, 1720±200, 1735±370, 1590±300 pg/ml at time points of 30, 60, 90, 120, and 150 minutes, respectively(n=6). At time point 180, antibody-included sample gave a value over that for the highest standard (2000 pg/mL) and thus could not be quantified. Control samples gave a collected value of 560±95, 260±85, 310 ±75, 480±55, 700±200, and 700 ±195pg/mL at time points of 30, 60, 90, 120, 150, and 180 minutes respectively(n=6). A significant difference was found between controls and antibody–included at time points of 60, 90, 120, and 150 minutes. These data are represented in Figure 3.2

### 3.3.4 In Vitro Microdialysis of IL-6

5 mL of 2ng/mL IL-6 was made up from stock in 10 mM aCEF. It was heated to 35ºC and stirred. CMA 20 PES probes were used to sample with a perfusion fluid of aECF. Samples were taken every 30 minutes during a three hour time period. Samples were stored at -20ºC and were quantified with BD opt EIA the following day. Average relative recoveries of 17.60% ±3.70% and 56.60%±22.70% were found for the control and antibody infused respectively(n=3). These data are represented in Figure 3.3.

### 3.3.5 In Vivo Microdialysis of IL-6

For the in vivo collection of IL-6 using the correct coordinates for the hippocampus, values of 279.77± 66.96, 351.79±38.78, 294.44±86.62, 271.44±161.88, 248.14±37.30, 211.37±30.06 pg/mL were found at time points of 30, 60,90, 120, 150, and 180 minutes respectivly for the control(n=3). Values of 257.06±40.41, 286.36±86.77, 364.18±240.04, 335.33±151.15, 434.46±82.09, 433.14±92.88 pg/mL were found for time points 30, 60, 90, 120, 150, and 180 minutes respectivly for the antibody-enhanced sample. These data are represented in Figure 3.4.

### 3.3.6 Comparison of Antibody enhanced Standards to Control

BD optEIA Rat MCP-1 ELISA kit (BD Biosciences, San Diego, CA) Standards were prepared in a solution that contained 1:500 dilution of the detection Antibody from the same kit in aCEF as well as assay diluent as per kit protocols. Both were then run according to kit protocols to see if the additional detection antibody had any effect on the assay. Results are graphically represented in Figure 3.5.

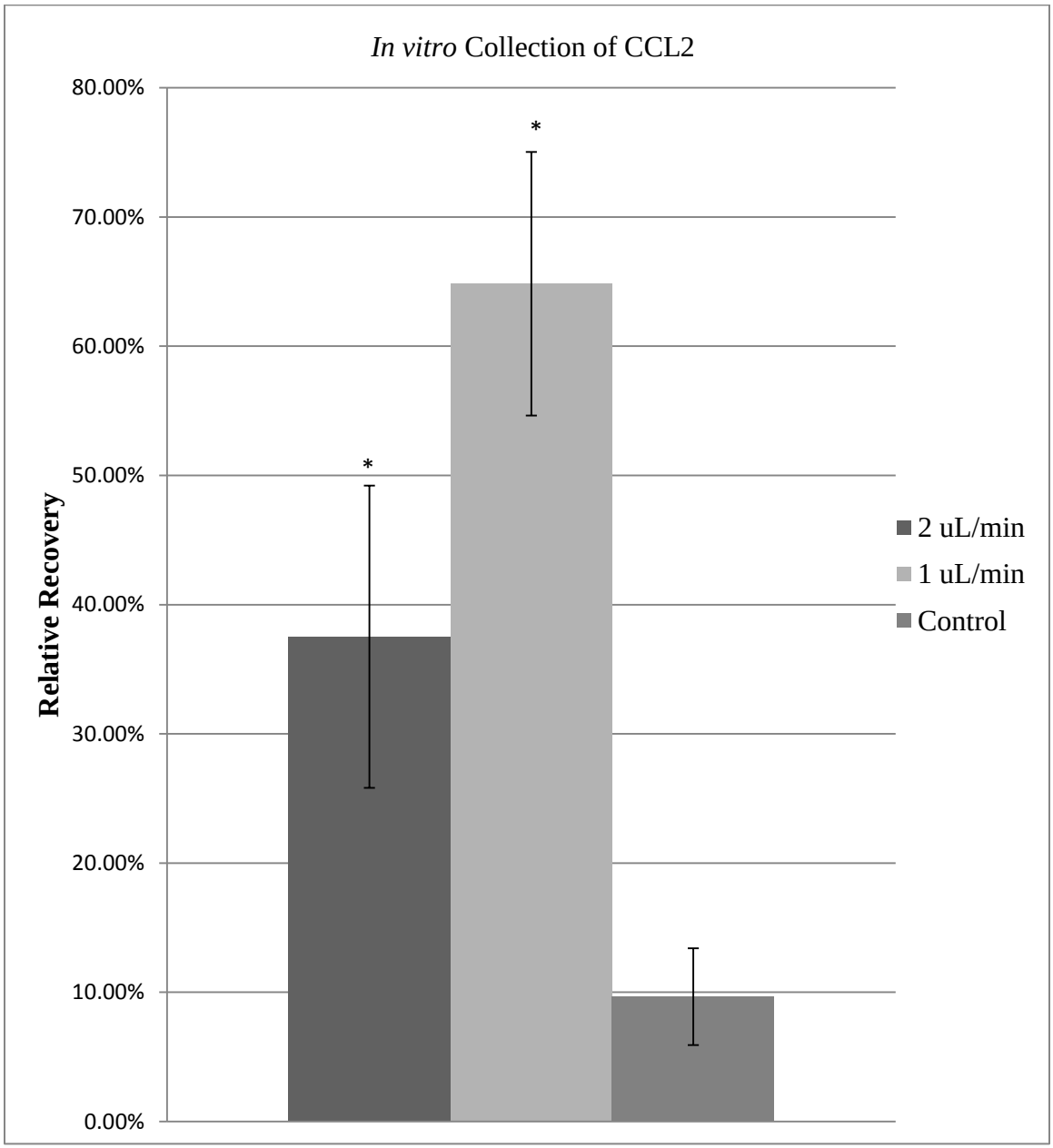



Figure 3.1: In vitro collection of CCL2. Error bars represent a standard deviation of n=10 in 2 µL/min and control, and n=5 in 1µL/min. * Denotes a significant difference to the control at the 95% confidence level.

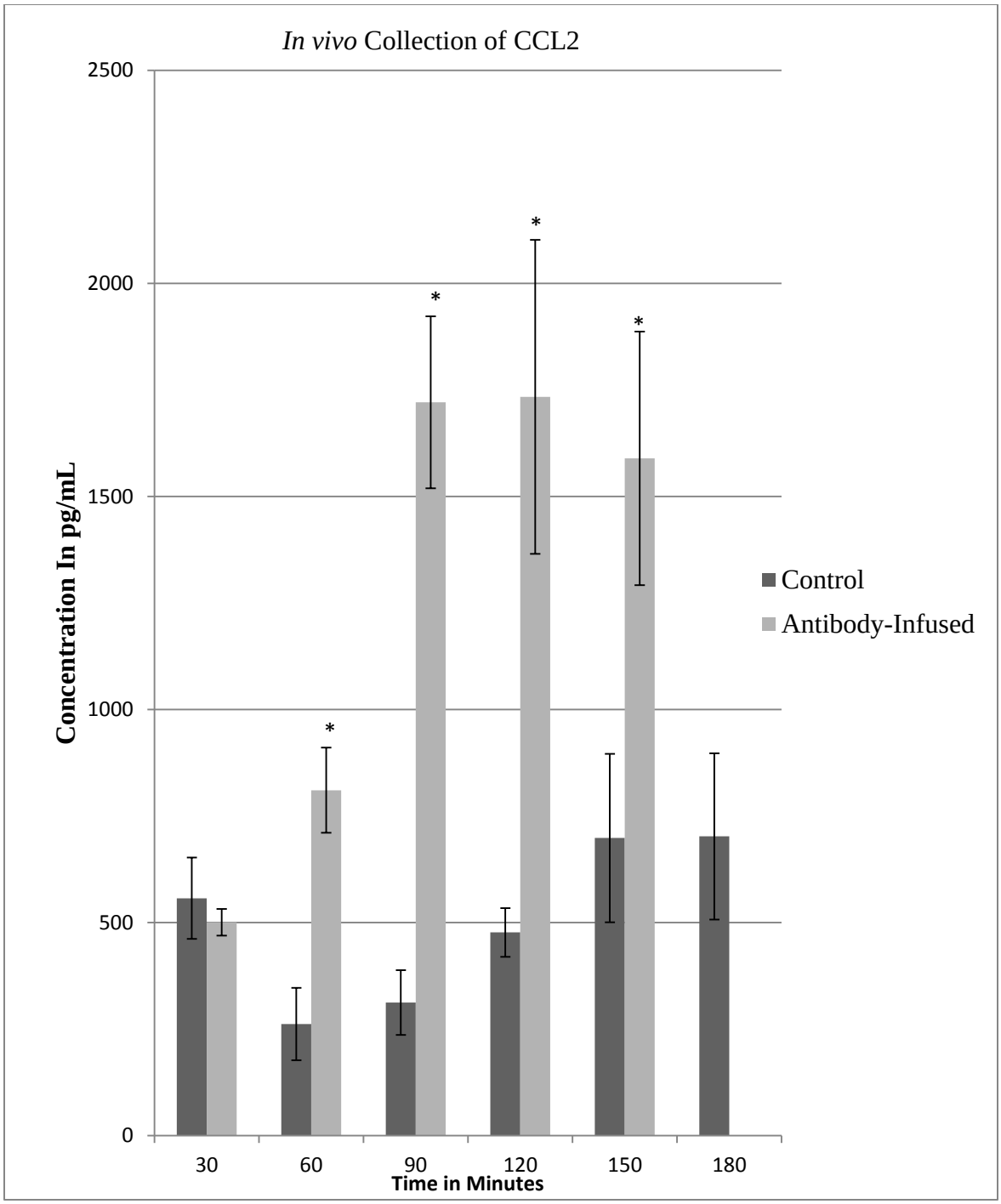



Figure 3.2: In vivo collection of CCL2. Error bars represent a standard deviation were n=6. A * denotes significant difference to the control at the 95% confidence level. Antibody-Infused data at time point 180 was over the range of standards so no quantitative value is reported.

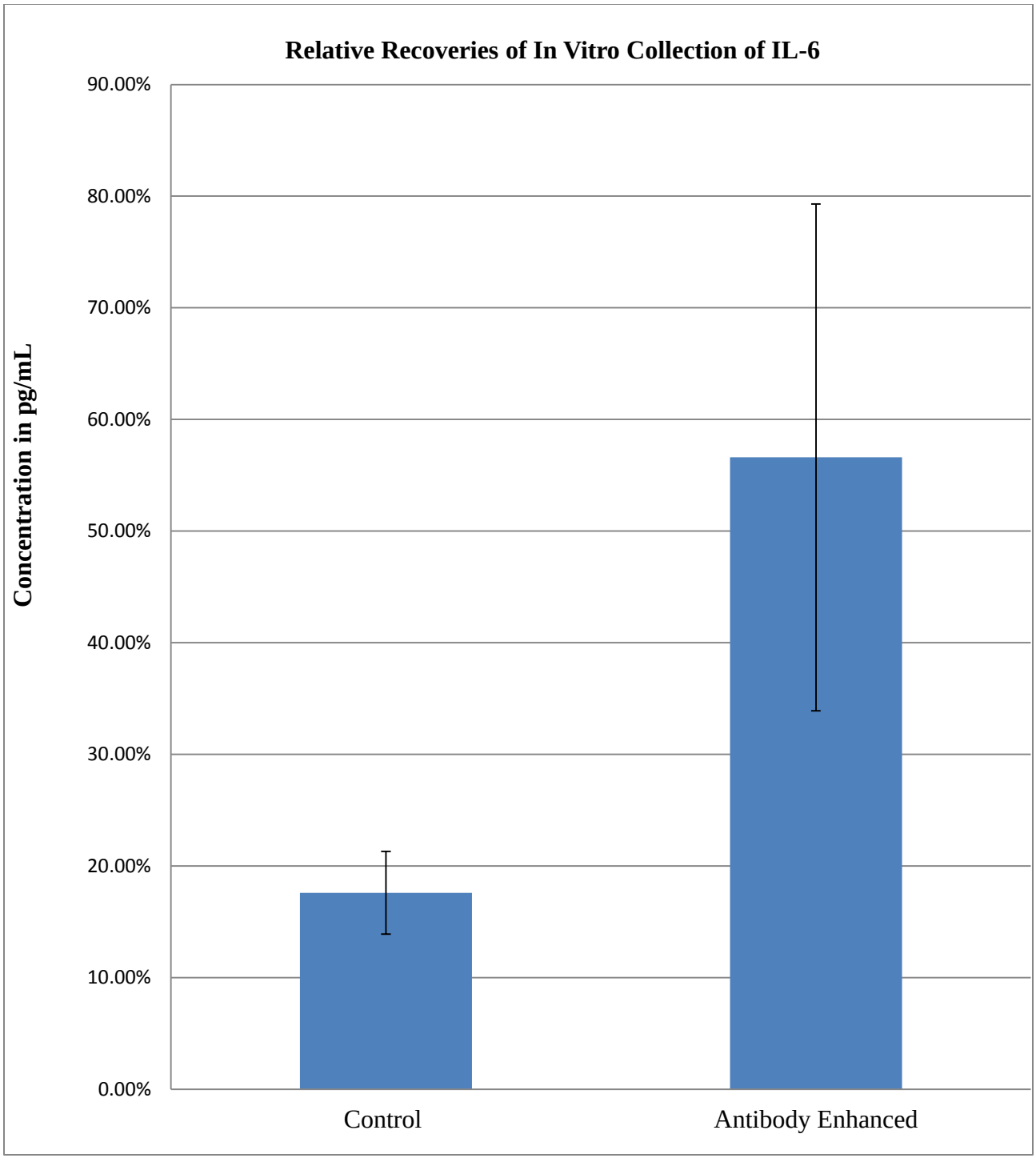



Figure 3.3 In vitro collection of IL-6. Values presented are an average were n=3. Error bars represent a standard deviation.

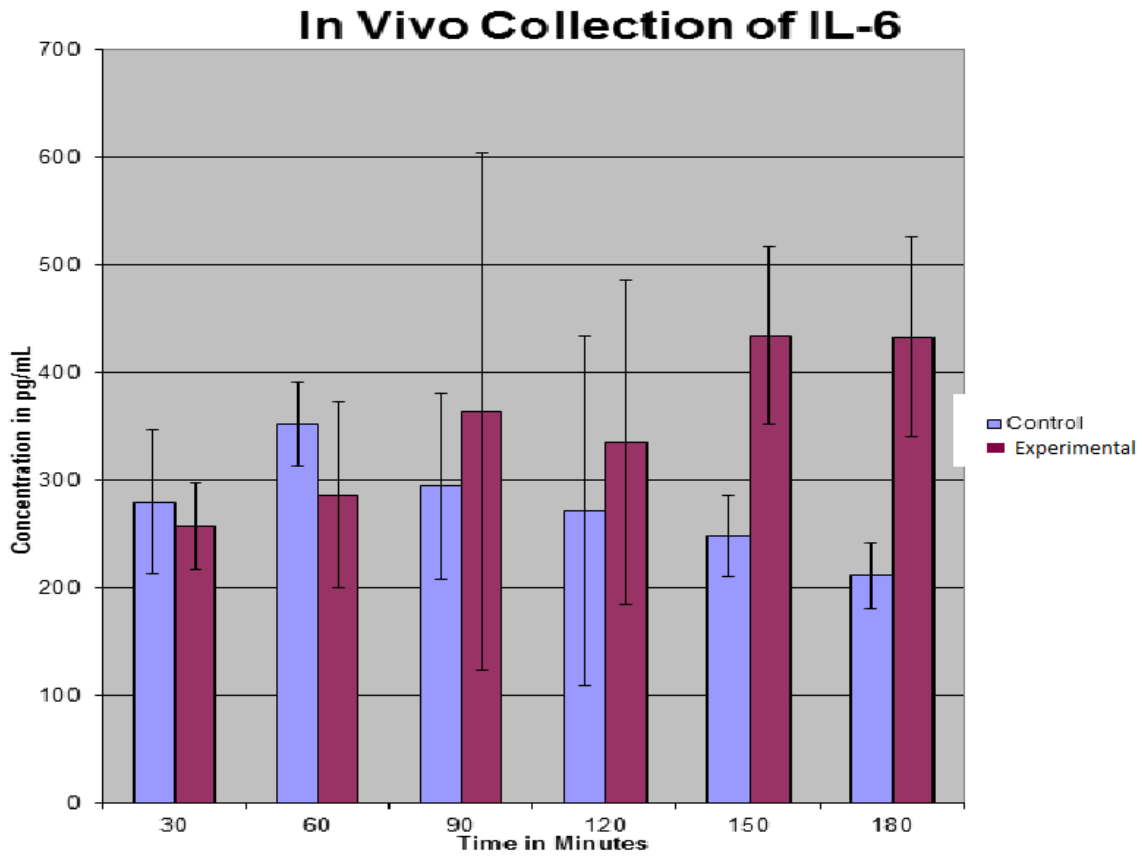



Figure 3.4 In vivo collection of IL-6. Values presented are an average of n=3 samples. Error bars represent a standard deviation.

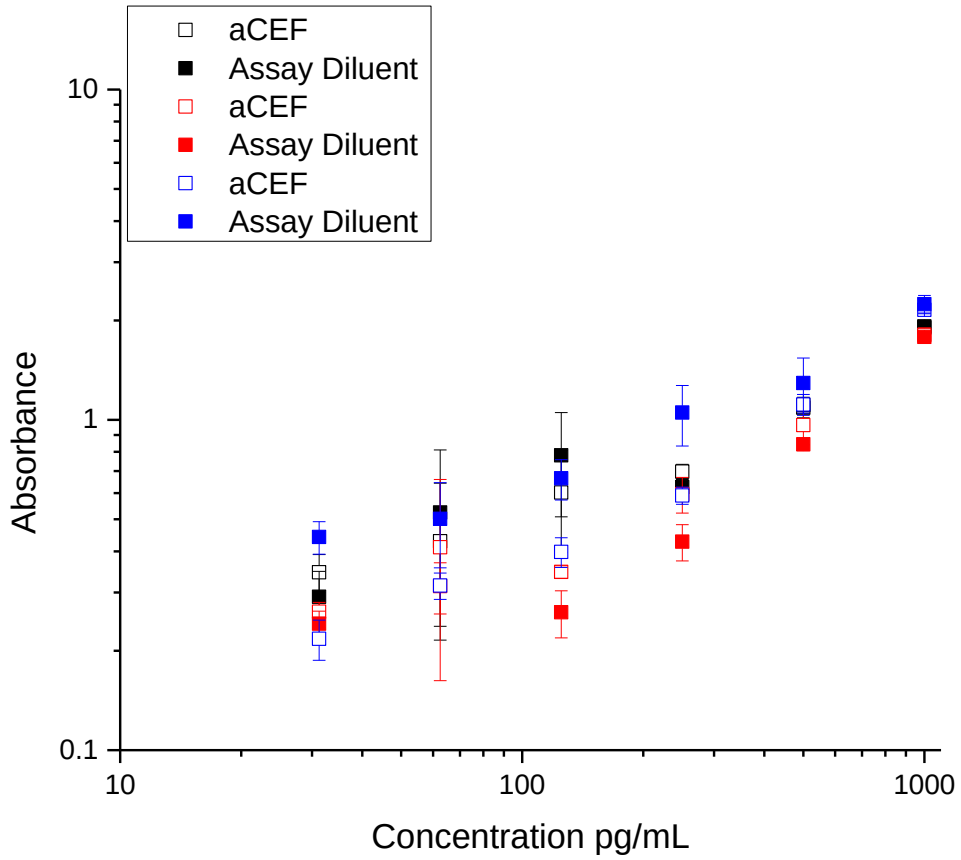



Figure 3.5 Comparison of Antibody added aCEF and Assay Diluent. Points are an average of (n=4). Error bars represent a Standard Deviation.

### 3.4 Discussion

In this work, we demonstrate the first usage of antibody-enhanced in vivo microdialysis sampling for the chemokine, CCL2 and IL-6. Pich et al. used antiserum against corticotropin-releasing factor and observed an approximate two-fold increase in their relative recovery.[13] This increase is most likely caused by the binding to the antibody which serves to increase the flux of the targeted analyte into the dialysis probe. For CCL2, we observed an approximate 3.5 fold increase in the *in vitro* relative recovery. This approximate 3.5 fold increase held for the *in vivo*

studies at the time points of 90, 120 and 150. This was somewhat unexpected since differences in the mass transfer properties between in vitro and in vivo experiments exist.

At the 30 minute time point, different scenarios could lead to the observed similar CCL2 concentrations. The first is that glial cells have been reported to contain MCP-1.[14, 15] In these studies, samples were immediately collected after probe insertion. This is because the rat was under isoflurane anesthesia and after several hours it becomes tricky to maintain rodents under this anesthetic. If the probe insertion caused significant release in the CCL2 from disrupted glial cells, it is likely the concentrations will be much higher in the collected dialysate before the protein is cleared. Additionally, it is important to remember that microdialysis sampling provides a time-averaged sample.[16] Depending on the time average, it is possible that concentrations could be similar between both dialysis probes. Then, after the initial injury, the dialysis probe may serve as a sink to help clear out some of the initially released cytokines. At this point, cytokines may be coming from local cells surrounding the dialysis probe resulting in much lower concentrations collected into the dialysate.

Once the initial cells have started to respond to the injury by producing their own cytokines increases in concentration are observed at 90, 120, 150 minutes. We have previously observed such increases in cytokines after implantation injury in the subcutaneous tissue.[17] At the 180 minute time point, the CCL2 concentration in the dialysate for the antibody-perfused probe was over the high end of the range (2000 pg/mL) and thus cannot be quantified.

Since this is a reported short communication, there is a significant amount of additional validation that would be necessary to more fully elucidate the biological events that are occurring after probe insertion. Furthermore, our long-term goal is to perform these studies in awake and

freely-moving animals and the concentrations from such studies may be very different than observed here.

In mouse homogenized brain, measured concentrations of CCL2 were reported as 50 pg/mg protein and 275 pg/mg protein at time points of 90 and 180 minutes respectively after a lipopolysaccharide (LPS) injection.[18] It is our understanding that in rodents, CCL2 has only been extracted from brain tissue via the use of homogenized tissues. Although the exact amount of brain volume from which this protein amount has been derived is unknown, the homogenized tissue CCL2 concentrations are presented as a general reference to compare the concentrations.

Recently, the collection of CCL2 from human brain has been reported from head trauma patients.[19] From these patients, median CCL2 values found in dialysates were reported to be between 2500 – 2550 pg/mL. For comparison, it is important to note that human microdialysis catheters have 10 mm long membranes compared to the 4-mm membranes used in our study. Additionally, flow rates of 0.3 µL/min were used in the Helmy et al. study vs. 2 µL/min in this study. Smaller membrane lengths and higher perfusion fluid flow rates would lead to lower relative recovery values and lower collected CCL2 concentrations reported for this study. Here we report CCL2 concentrations in control dialysates between 260 and 700 pg/mL and antibody enhanced values as high as 1735 pg/mL throughout the experimental collection period.
For IL-6 no significant difference was found between the Antibody enhanced and control collection in both the *in vitro* and *in vivo*. In both of the *in vitro* and *in vivo* an average increase is seen in the antibody enhanced and control however variation is too large in the antibody enhanced to state that there is a significant difference between the two. In the *in vitro* data re report an average almost two times greater than that of the control. In the *in vivo* at initial time points there appears to be no difference between antibody enhanced and control. At later time

points the average in the antibody enhanced starts to increase over the control to be again two times that of the control. Again the variation was too large in the antibody enhanced to determine a significant difference though. Winter et al report a relative recovery of 45.29± 8.4% for in vitro collection of IL-6, in comparison to our 17.6%.[20] The differences in the probe length, membrane molecular weight cut off, and flow rate would explain this difference. The length used by Winter et al was 12-15mm in compared to the 4mm length of the probe used in these data, the molecular weight cut off was smaller at 3000 kDa in comparison to 10,000 kDa in these data, and the flow rate Winter et al. used was 1µL/min in comparison to 2 µL/min that was used for these data, all of these would contribute to a larger relative recovery of IL-6 by Winter et al. *In vivo* Winter et al report a max value of 800 pg/mL for IL-6 from human brain tissue.[20] Godbout et al. report a basal concentration of about 100 pg/mL in mouse were we report a concentration of about 250 pg/mL at similar time points.[21] There are many possible reasons for these differences between our values and those of others including the foreign body or wounding response to the probe, the difference in species, and other methodological differences between the different studies.

The optimization of the use of a detection antibody in the perfusion fluid allowed for straight-forward measurement of the samples using a standard ELISA plate and kit. This initial work presented here paves the way for trying the antibody-enhancement approach to many other soluble protein targets in the brain ECF.

One concern when these data were presented was the concern that the extra detection antibody in the samples may have had an effect on the assay. To test this theory standard were prepared in two solutions. The first was a 1:500 dilution of the detection antibody in aCEF and the second was the assay diluent that is kit protocol for the standards. The results showed that

for CCL2 there was no significant difference between the standards prepared with the 1:500 dilution of antibody to those of just assay diluent.

### 3.5 References


1. Shen, Y.-Q.; Hebert, G.; Lin, L.-Y.; Luo, Y.-L.; Moze, E.; Li, K.-S.; Neveu, P. J., Interleukin-1beta and interleukin-6 levels in striatum and other brain structures after MPTP treatment: influence of behavioral lateralization. *J. Neuroimmunol.* 2005, 158, 14-25.

2. Signore, A.; Mather, S. J.; Piaggio, G.; Malviya, G.; Dierckx, R. A., Molecular imaging of inflammation/infection: Nuclear medicine and optical imaging agents and methods. *Chem. Rev.* 2010, 110, 3112-3145.

3. Maier, T.; Gueell, M.; Serrano, L., Correlation of mRNA and protein in complex biological samples. *FEBS Lett.* 2009, 583, 3966-3973.

4. Greenbaum, D.; Jansen, R.; Gerstein, M., Analysis of mRNA expression and protein abundance data: an approach for the comparison of the enrichment of features in the cellular population of proteins and transcripts. *Bioinformatics* 2002, 18, 585-596.

5. Helmy, A.; Carpenter Keri, L. H.; Skepper Jeremy, N.; Kirkpatrick Peter, J.; Pickard John, D.; Hutchinson Peter, J., Microdialysis of cytokines: methodological considerations, scanning electron microscopy, and determination of relative recovery. *J. Neurotrauma* 2009, 26, 549-61.

6. Mellergard, P.; Aneman, O.; Sjogren, F.; Pettersson, P.; Hillman, J., Changes in extracellular concentrations of some cytokines, chemokines, and neurotrophic factors after insertion of intracerebral microdialysis catheters in neurosurgical patients. *Neurosurgery* 2008, 62, 151-7; discussion 157-8.

7. Maurer, M. H.; Haux, D.; Unterberg, A. W.; Sakowitz, O. W., Proteomics of human cerebral microdialysate: from detection of biomarkers to clinical application. *Proteomics Clin. Appl.* 2008, 2, 437-443.

8. Woodroofe, M. N.; Sarna, G. S.; Wadhwa, M.; Hayes, G. M.; Loughlin, A. J.; Tinker, A.; Cuzner, M. L., Detection of interleukin-1 and interleukin-6 in adult rat brain, following mechanical injury, by in vivo microdialysis: evidence of a role for microglia in cytokine production. *J. Neuroimmunol.* 1991, 33, 227-36.

9. Ao, X.; Stenken, J. A., Microdialysis sampling of cytokines. *Methods* 2006, 38, 331-341.

10. Fletcher, H. J.; Stenken, J. A., An in vitro comparison of microdialysis relative recovery of Met- and Leu-enkephalin using cyclodextrins and antibodies as affinity agents. *Anal. Chim. Acta* 2008, 620, 170-175.

11. Duo, J.; Fletcher, H.; Stenken, J. A., Natural and synthetic affinity agents as microdialysis sampling mass transport enhancers: Current progress and future perspectives. *Biosens. Bioelectron.* 2006, 22, 449-457.

12. McNay, E. C.; Sherwin, R. S., From artificial cerebro-spinal fluid (aCSF) to artificial extracellular fluid (aECF): microdialysis perfusate composition effects on in vivo brain ECF glucose measurements. *J. Neurosci. Methods* 2004, 132, 35-43.

13. Pich, E. M.; Koob, G. F.; Heilig, M.; Menzaghi, F.; Vale, W.; Weiss, F., Corticotropin-releasing factor release from the mediobasal hypothalamus of the rat as measured by microdialysis. *Neuroscience* 1993, 55, 695-707.

14. Magge, S. N.; Malik, S. Z.; Royo, N. C.; Chen, H. I.; Yu, L.; Snyder, E. Y.; O'Rourke, D. M.; Watson, D. J., Role of monocyte chemoattractant protein-1 (MCP-1/CCL2) in migration of neural progenitor cells toward glial tumors. *J. Neurosci. Res.* 2009, 87, 1547-1555.

15. Hayashi, M.; Luo, Y.; Laning, J.; Strieter, R. M.; Dorf, M. E., Production and function of monocyte chemoattractant protein-1 and other β-chemokines in murine glial cells. *J. Neuroimmunol.* 1995, 60, 143-50.

16. Ståhle, L., Pharmacokinetic Estimations from Microdialysis Data. *European Journal of Clinical Pharmacology* 1992, 43, 289-94.

17. Wang, X.; Lennartz, M. R.; Loegering, D. J.; Stenken, J. A., Interleukin-6 Collection through Long-Term Implanted Microdialysis Sampling Probes in Rat Subcutaneous Space. *Analytical Chemistry* 2007, 79, 1816-24.

18. Thompson, W.; Karpus, W.; Van Eldik, L., MCP-1-deficient mice show reduced neuroinflammatory responses and increased peripheral inflammatory responses to peripheral endotoxin insult. *J. Neuroinflammation* 2008, 5, 35.

19. Helmy, A.; Carpenter, K. L. H.; Menon, D. K.; Pickard, J. D.; Hutchinson, P. J. A., The cytokine response to human traumatic brain injury: temporal profiles and evidence for cerebral parenchymal production. *J. Cereb. Blood Flow Metab.* 2011, 31, 658-670.

20. Winter, C.; Iannotti, F.; Pringle, A.; Trikkas, C.; Clough, G.; Church, M., A microdialysis method for the recovery of IL-1 beta, IL-6 and nerve growth factor from human brain in vivo. *Journal of Neuroscience Methods* 2002, 119, 45-50.

21. Godbout, J. P.; Berg, B. M.; Kelley, K. W.; Johnson, R. W., [alpha]-Tocopherol reduces lipopolysaccharide-induced peroxide radical formation and interleukin-6 secretion in primary murine microglia and in brain. *Journal of Neuroimmunology* 2004, 149, 101-109.

## 3.6 Appendix

Parts of this chapter are reprinted with permission from Elsevier

| | |
|---|---|
| Order Number: | 500619264 |
| Publication: | Journal of Neuroscience Methods |
| Title: | Antibody-enhanced microdialysis collection of CCL2 from rat brain |
| Type of Use: | Thesis/Dissertation |

UNIVERSITY OF ARKANSAS

J. William Fulbright College of Arts and Sciences
*Chemistry and Biochemistry*

Patricia Koski, Ph.D.
Associate Dean, Graduate School & International Education
University of Arkansas

Dear Dean Koski:

With this letter, I certify that Mr. Anthony Herbaugh is the first co-author on the paper that constitutes Chapter 3 in his M.S. thesis. He has completed at least 51% of the work on this published manuscript. A public citation for this manuscript may be found on PubMed (http://www.ncbi.nlm.nih.gov/pubmed/). That citation reads as follows:

Antibody-enhanced microdialysis collection of CCL2 from rat brain.
Herbaugh AW, Stenken JA.J Neurosci Methods. 2011 Nov 15;202(2):124-7.
doi: 10.1016/j.jneumeth.2011.05.006. Epub 2011 May 11.PMID:21600925

Please feel free to contact me if there are additional concerns about effort for this particular chapter of Mr. Herbaugh's thesis.

Sincerely,

Julie A. Stenken, Ph.D.
Professor
Chemistry and Biochemistry
(479) 575-7018

CHEM 119 • 1 University of Arkansas • Fayetteville, AR 72701-1201 • 479-575-4601 • Fax: 479-575-4049 • http://chemistry.uark.edu/

## 4.0 Development of an In-House Created CCL2 Bead Assay

### 4.1 Introduction

Detection of multiple cytokines with in the same sample is of a discernible interest for comparison studies. Such comparison studies could give an insight into the greater understanding of any possible regulatory roles that cytokines may have on other neurological functions as well as wound healing. The ability to multiplex samples would reduce the number of samples needed. It could possibly eliminate run to run errors in comparison studies of two corresponding intervals when you are looking for neuropeptides. For these experiments a flow cytometry system was used that has allowed for the multiplexing of cytokines. The method of this flow cytometry was discussed in Chapter 1. In quick summary the Luminex method allows the use of different bead types. These bead types have different antibody attached to them. The Luminex instrument is able to differentiate between these bead types, thus allowing for the use of several types of beads at the same time. Each bead type could be a different cytokine being analyzed.

In these experiments it will attempted to mimic the attachment chemistry used in commercial beads that have bound cytokine antibodies. Determination and successful mimicry of this chemistry will allow us to attach cytokine antibodies to the beads. This will allow us to develop multiplexed assays of cytokines that may not have a commercially available kit.

## 4.2 Materials and Methods

### 4.2.1 Materials

Luminex xMAP carboxylated microspheres (Austin, TX) Bead set 160. Phycoerythrin conjugated F(ab')2 fragment of affinity purified anti-rabbit IgG from Donkey purchased from Rockland (Gilbertsville, PA). Bovine serum albumin, 2[N-morpholino]ethansulfonic acid (MES) and sodium azide were purchased from Sigma (St. Louis, MO). Sodium chloride, potassium chloride, sodium phosphate monobasic and TWEEN-20 from Fisher Scientific (Pittsburgh, PA). Polylink 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) was purchased from Polysciences, Inc. (Warrington, PA). Sulfosuccinimidyl-6-[biotin-amido]hexanoate (sulfo-NHS-LC-biotin), N-hydroxysuccinimide (NHS) and EZ Biotin Quantitation Kit were purchased from Pierce (Rockford, IL). Streptavidin, R-phycoerythrin conjugate (SAPE) was purchased from Invitrogen (Eugene, OR) Activation buffer -0.1M sodium phosphate monobasic, pH 6.2, Coupling buffer - 0.05M 2-(Nmorpholino) ethanesulfonic acid (MES), pH 5.0, PBS-TBN blocking/storage buffer for beads - PBS, 0.1% BSA, 0.05% azide, pH 7.4, Assay buffer - PBS, 1% BSA, pH 7.4, Wash buffer - PBS, 0.05% TWEEN-20, pH 7.4. PBS buffers all contain 138 mM NaCl and 2.7 mM KCl. Multiscreen HTS, BV 96-well filter plates were purchased from Millipore (Billerics, MA). Purified mouse anti-rat MCP-1 (Cat# 555072) and Biotin Mouse anti-Rat MCP-1(Cat# 555074) were purchased from BD Biosciences Pharmigen. Milliplex Map Rat Cytokine/Chemokine Immunoassay (Cat# RCYTO-80k-04) was purchased from Millipore.

### 4.2.2 Instruments

Luminex$^{100}$ IS (Luminex), ISS-PC1 photon counting spectroflourometer (Vinci).

### 4.2.3 Bead Preperation

The stock beads were resuspended by vortex and sonicated. Next, 400 µL of stock beads were transferred into a 1.5 mL microcentrifuge tube and pelleted at 10000 x g for 2 minutes. The supernatant was removed and the beads were resuspended in 100 µL of HLPC grade water, vortexed and pelleted. The supernatant was removed and 80 µL of 100 mM monobasic sodium phosphate, pH 6.2 was added and the beads were vortexed. Next 10 µL of 50 mg/mL of NHS (diluted in HLPC water) was added and the beads were vortexed. Then, 10 µL of 50 mg/mL EDC (diluted in HPLC water) was added and the beads were vortexed. The beads were then incubated for 20 minutes with 10 minutes intervals of mixing. The activated beads were centrifuged, supernatant removed and 250 µL of 50 mM MES, pH 5.0 was added. The beads were vortexed and pelleted and supernatant removed. The beads were washed again using MES for a total of 2 washes. Next, the beads were resuspended in 100 µL of 50 mM MES and vortexed. Then 10 µL of stock antibody was added and 390 µL of 50 mM MES was added to bring the total volume up to 500 µL. The reaction proceeded on a plate shaker at room temperature for 2 hours. Next, the beads were pelleted, supernatant removed and the beads were vortexed in 1 mL of PBS buffer. The beads were pelleted, supernatant removed and washed again with PBS buffer for a total of 2 washes. Finally, the beads were resuspended in 1 mL of PBS- buffer and counted using a hemocytometer.

### 4.2.4 Protocol for Sandwich Immunoassay

The beads were resuspended by vortex and sonication for 20 seconds. A working bead mixture was prepared by diluting the coupled beads to a final concentration of 100 beads/µL in assay buffer. A 96-well filter plate was pre-wetted and 25 µL of assay buffer was added to each well + 25 µL of sample + 25 µL of beads. The plate was covered with foil and allowed to mix on a plate shaker overnight at 4ºC. Next, the plate was washed 3X using wash buffer and 50 µL of 10 µg/mL of detection antibody was added to was well. The plate was incubated at room temperature on a shaker for 2 hours. The plate was washed again 3X with wash buffer and 100 µg/mL of SAPE was added to each well and the plate incubated on the shaker for 1 hour. The plate was washed a final time (3X) using wash buffer and then 100 µL of sheath fluid was added to each well for analysis. The plate was then analyzed using the Luminex system.

**4.3 Results**

For the commercially available kit an average of Mean Fluorescence Intensity(MFI) of 12734±132, 11375±105, 6198±86, 699±37, 78±8, 10±3, 4±2 are reported for concentrations of 20,000, 5000, 1250, 312.5, 78.13, 19.53, and 4.88pg/mL of MCP-1 respectively were n=3. For the home made assay MFIs of 8328±94, 7982±103, 3712±56, 146±12, 62±9, 10±3, 5±2 are reported for concentrations 20,000, 5000, 1250, 312.5, 78.13, 19.53, and 4.88pg/mL of MCP-1 respectively were n=3. This data is shown in Figure 4.1.

The homemade kit shows a significant decrease in MFI at the higher levels of concentration of 20,000, 5000, 1250, and 312.5pg/mL. However there is no significant difference at the 78.13, 19.53, and 4.88pg/mL concentrations.

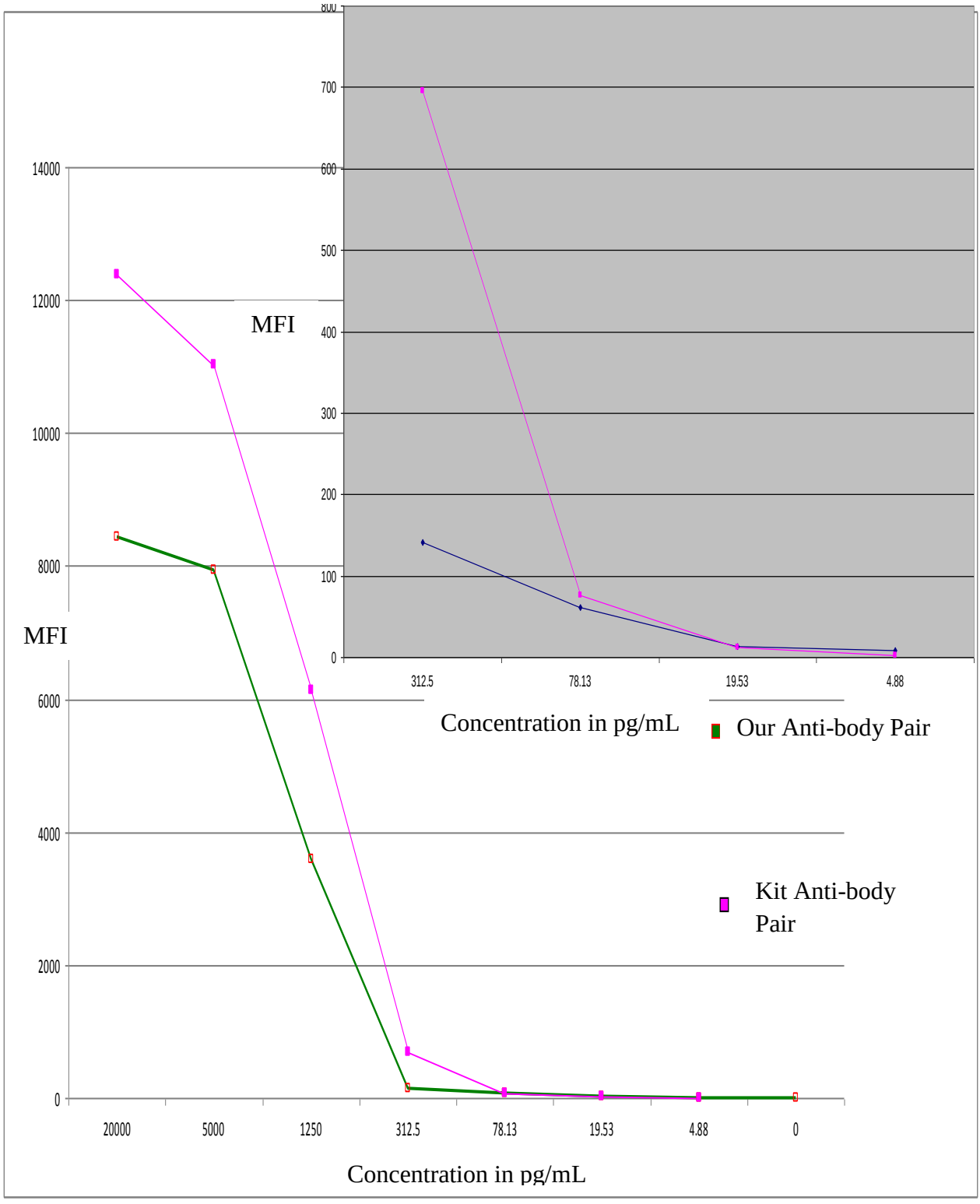



Figure 4.1 Comparison of MFIs (n=3) of decreasing concentrations of MCP-1 from commercially bought Luminex kit vs Home Made kit.

## 4.4 Discussion

In these experiments it was attempted to create a homemade fluorescence sandwich assay. It was believed that variation was caused by the difference in the antibody pairs that were used. This different binding affinity could cause the variation in the level of attachment and thus MFI. The commercially used antibodies clones were considered a trade secret and thus true comparison with the same antibody clone could not be done. However, results could also be evidence of better attachment of the capture antibody in the commercially available assay. Comparison of the amount of attachment could not be performed as the detection of the C-terminus of the capture antibody was attached to the bead. Thus, a detection antibody of the capture antibody was unable to be used. Further studies can be done to insure that this attachment method works properly for the attachment of similar animal antibodies developed for cytokines.

## 5.0 Conclusions

As mentioned in chapter one, cytokines have been linked to neurological diseases including, but not limited to Huntington's, alcoholism, and HIV dementia. Cytokines have also been found to have a correlation and possibly causation in several neurological disorders including Epilepsy and Cerebral Infarct (Stroke). It is important to know that many of the functions of cytokines are concentration dependent. Changes in the concentration of cytokines can reveal stages of wound development or the presence of a disease state. Because of this a method of detecting cytokine concentration over times is needed. Microdialysis is a method that allows the collection of cytokines from the target area and allows for a time lapse analysis of changes in concentration. However microdialysis has limitations in collecting cytokines, the work presented in this thesis attempts to overcome some of those limitations and develop methods that will allow for a wider use of microdialysis in clinical treatment studies.

One of these limitations is that cytokines have a low relative recovery. This presents a problem in detection and detection methods. Cytokines are in low concentrations *in vivo*, about the low pico- molar range. Low relative recovery means that even less cytokine is present in the sample which may make detection and quantification difficult. In chapter two, experiments to develop new methods of cytokine collection using microdialysis are presented. In these experiments a method termed Stopped Flow are used to try and improve the relative recovery. In Stopped Flow microdialysis the perfusion fluid is stopped for a set time to allow for a longer residence time. This increase in residence time it was hypothesized increase the relative recovery.

In these experiments however it was found that while an average increase in the relative recovery was gained. A significant difference could not be determined because of the large variation. An experiment using methyl orange was then done to see if the variation could be reduced. In the methyl orange collection a significant difference was seen in the stopped flow from the continuous flow at the 95% confidence interval. With these data it is believe that the variation in the cytokine stopped flow experiment was due to the molecule size and the tortious path that it had to travel to get through the membrane. Valuable information about the diffusion of the molecules and the nature and relation of the membrane/cytokine interaction is shown and could lead in future research to a greater understanding of this complex process of microdialysis collection. In this study we attempt to improve the collection by manipulating the parameters of the flow alone. Further research could focus on different intervals as well as different collection flow rates. These experiments could identify a variation that allowed for a greater collection of cytokines without the addition of affinity agents.

Previous studies have improved the collection by adding affinity agents, such as Heparin to the perfusion fluid.[1] In chapter three a method of collection of cytokines from *in vivo* brain tissue using antibodies as affinity agents is presented. The use of antibodies as affinity agents increases the relative recovery by reducing the amount of free agent in the perfusion fluid. By binding the analyte as it enters the perfusion fluid equilibrium forces drive more into the perfusion fluid. In these studies a greater recovery of cytokines was found using the antibody affinity agents. Free antibody used in the perfusion fluid increased the relative recovery of CCL2 by 3 fold in both *in vitro* and *in vivo*. Free antibody in the perfusion fluid showed an increase in the average relative recovery of IL-6 however the variation was too large to say that this was a significant difference in both *in vitro* and *in vivo*. Experiments were done to see if the free

antibody in the perfusion fluid caused interference with the ELISA. Data presented here in this thesis show that at the 95% confidence interval free antibody in the perfusion fluid does not have a significant difference than the assay diluent used by the kits.

This study however, was limited by the time of the rat survival under anesthesia. In further studies the animal would be awake and free to move allowing for a much longer collection period that extending into days. In addition, further studies could look into the collection of neuropeptides as well as cytokines, using the antibody affinity agent method. Work done on inhibition of the production of cytokines[2], could also be used as treatment experiments. The implication and uses of this method extends beyond the scope this discussion. They cannot all be fully discussed here due to numerous options. Refinement of this method may lead to the inclusion of the affinity agent method into clinical trials that are already being conducted with microdialysis[3].

In chapter four a method for the production of bead based assays is presented. These bead based assays are of interest for current cytokine research because of the ability to multiplex with them. Since the cytokine network has many factors that regulate it is of great interest to be able to be able to quantify many of them in the same sample. There are currently commercially available kits for multiplexing several cytokines. However some of the cytokines are not available as well as some of the smaller molecules that are of interest in wound healing and neurological disease states. In chapter four a method is developed to create beads that have antibodies for these cytokines that do not have current commercially available kit or these smaller molecules that do not have a sandwich assay available. These data presented in chapter four shows that using this attachment chemistry can be used to make beads that are similar to commercially available kits. Further development of this attachment and use of beads could lead

to the production of a competitive assay for molecules that are too small for the possibility of sandwich assays.[4]

In final conclusion the experiments presented here address some of the major concerns with microdialysis sampling of cytokines. The research presented provides insight and methods to improve future work done in the collection of cytokines with microdialysis. This work can eventually lead to the clinical use of microdialysis as a means of marker identification of disease states, wound healing response control, neuro-communication understanding, and many other fields that go beyond our current understanding and use of microdialysis.

### 5.1 References


1. Duo, J. Affinity Microdialysis Sampling of Cytokines. Rensselaer Polytechnic Institute, Troy, New York, 2009.

2. Ahmed, F.; Tessarollo, L.; Thiele, C.; Mocchetti, I., Brain-derived neurotrophic factor modulates expression of chemokine receptors in the brain. *Brain Res.* 2008, 1227, 1-11.

3. Benveniste, H.; Hansen, A. J., Practical aspects of using microdialysis for determination of brain interstitial concentrations. In *Microdialysis in the Neurosciences*, Elsevier, Amsterdam, 1991; Vol. 7, pp 82-102.

4. Fletcher, H. J.; Stenken, J. A., An in vitro comparison of microdialysis relative recovery of Met- and Leu-enkephalin using cyclodextrins and antibodies as affinity agents. *Analytica Chimica Acta* 2008, 620, 170-175.